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Factorial experimental design and principal component analysis of the interaction of animal glues with polymeric and silica-based stationary phases in size exclusion chromatography

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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 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. The propensity toward ion exclusion is greater on the silica-based stationary phase. Hydrophobic adsorption of collagen and Croda glue was evident on the polymeric phase. Collagen and Croda glue display identical behaviour on both stationary phases as suggested by their principal components. Anomalous behaviour of VM gelatin is suggested by both main factor coefficients and principal component analysis.

1. Introduction

Animal glues, gelatins and other natural organic additives have been employed for decades in the base metals refining industry as levelling agents in the electrodeposition of copper, lead and zinc from acidic sulphate solutions. The net effect of these additives is to provide a smooth metal deposit with a preferred, compact crystal orientation at a high current efficiency. The effects of glue and/or gelatin additives on the current efficiency of zinc electrolysis is well documented [1–7]. However, the exact mechanism and the molecular species responsible for causing these effects have yet to be determined.

Size exclusion chromatographic (SEC) techniques are commonly employed to analyze and purify biomolecules, such as glues and gelatins, according to their molecular size (Stokes radius) or molecular mass. Animal glues and gelatins are derivatives of Type I collagen and have molecular masses between 94 000 and 292 000. Analysis of Croda glue (an animal glue common to the base metals industry) by SEC however, illustrated a molecular mass of 491 000 [2]. This discrepancy between the known molecular masses of collagen and the measured molecular mass of Croda glue is possibly related to non-ideal size exclusion effects. The polymeric stationary

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phases employed in SEC are known to exhibit tendencies towards ion exclusion and hydrophobic adsorption [8–10]. Furthermore, collagen tends to adsorb to SEC stationary phases through ion exchange or hydrophobic interaction [11].

The molecular masses of biomolecules with symmetrical conformations are easily determined, in the absence of secondary retention, as a direct correlation between the molecular size and the molecular mass of symmetrical molecules exists [12]. Similar relationships also exist for biomolecules which assume conformations which are not symmetrical, such as random coils or rod-like conformations. However, these relationships cannot be applied to the determination of the molecular masses of these species in denaturing eluents containing SDS [13–15]. The accurate measurement of apparent molecular mass (M_r) for proteins by SEC is further complicated by ionic and hydrophobic interactions of these solutes with the stationary phase [10,16–21]. The nature and extent of these interactions depend largely upon the type of stationary phase and the eluting conditions employed. Porous silica stationary phases contain large numbers of silanol groups which may interact with solutes via ion exclusion, hydrogen bonding or ion exchange. Derivatization of silica stationary phases does not deter ionic interactions due to their long-range nature [22]. Polymeric stationary phases such as that employed in this study, TSKGel PW, are hydrophilic, cross-linked polyether gels containing residual carboxyl groups [10,22]. Ion exclusion effects are therefore also expected with this type of stationary phase. Several theoretical approaches to account for ionic interactions have been attempted [9,20,23,24], yet problems with their universal application still exist. The determination and understanding of non-ideal SEC effects is probably the greatest deterrent to the development of an accurate theoretical model for SEC [11]. Clearly, a careful evaluation of potential solute–stationary phase interactions must be undertaken before accurate measurement of M_r for uncharacterized biopolymers can be achieved.

Type I collagen molecules display cationic character below pH 4–5 [2,25,26]. Since the efficient separation of cationic polyions on conventional silica-based stationary phases has yet to be reported [10,22] 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 conformations [27–29]. Denaturing conditions also increase resolution and in some cases the accuracy of the determination of M_r [30].

Factorial experimental design (FED) is useful to obtain an enhanced mechanistic understanding of chromatographic processes [31,32]. It is also a useful tool to map the responses of a given system in a multi-factor environment while minimizing the number of required experiments [31,33–35]. Furthermore, factor–factor interactions, which are almost always ignored when designing SEC and other chromatographic separation strategies [33–35], are readily determined. In order to facilitate the evaluation of solute–stationary phase interactions, a FED approach is employed in this work for the SEC of industrial glues and gelatins under denaturing conditions. Where numerous different types of solute–stationary phase interactions may lead to mixed modes of SEC separations, FED should clarify the nature and extent of these interactions. A Type I collagen of similar composition to these additives is also examined as a control. A three-factor two-level FED (2^3) is employed in an effort to gauge the magnitude of exclusion, adsorption effects and any first order interaction effects [36] on a polymeric stationary phase. The FED is also applied to a silica-based stationary phase since its mechanism of macromolecular separations is apparently not similar to that of the polymeric stationary phase [37]. A comparison of the results for the two stationary phases should give a clear indication of the type and magnitude of any effects, other than size exclusion, that influence the separation of collagen based protein–SDS complexes. The stationary phases are further characterized with respect to

the contributions of secondary effects to the separation process by coupling principal component analysis (PCA) to FED.

2. Experimental

2.1. Reagents

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

2.2. Apparatus

A Dionex chromatography system (Sunnyvale, CA, USA) was used for all experiments. It consisted of an APM analytical pump, VDM-II variable wavelength UV–vis detector and an LCM-I chromatography module. The injection valve was equipped with a 100 μl sample loop. Data was collected with the CIM-I chromatographic interface module using the AI-450 software (Version 3.31) with a custom built IBM style 386 host computer. A BioSil SEC-250 (BioRad Laboratories, Mississauga, Ontario, Canada) silica-based column (30 cm × 7.8 mm I.D.) and a guard column (5 cm × 7.8 mm I.D.) were used as a silica stationary phase. A TosoHaas TSK Gel 4000PW$_{xL}$ (Supelco, Oakville, Ontario, Canada) column (30 cm × 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 in an output range of 0.005 AUFS. All statistical calculations were performed using Microsoft EXCEL 4.0 (Redmond, WA, USA).

2.3. Procedure

Blue dextran and the nitrate anion were used to determine the void volume and the total column volume, respectively. Fresh stock solutions of Croda glue and VM gelatin were prepared every 3 days by stirring 0.100 ± 0.001 g at 80°C for 20 min in water followed by cooling at ambient temperature for 2 h. The solutions were then bulked up to 100 ml to give a concentration of 1.0 g/l, 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 10–15 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 errors.

3. Results and discussion

Three factors are considered for the FED: pH, methanol (CH_3OH) concentration and ionic strength (*I*). The factor levels for this study are shown in Table 1. The concentration of SDS is not considered as a factor since literature data have shown that protein–SDS binding ratios do not change significantly with SDS concentration in the ionic strength range 0.05–0.5 *M* [27,38]. Moreover, the conformation of the collagen–SDS complexes is not appreciably influenced by pH at the SDS concentration (3.5 m*M*) em-

Table 1
Factorial experimental design parameters

Factor	Low	High
pH	4	7
[CH_3OH]	0% (v/v)	15% (v/v)
I (Ionic strength)	0.10 M	0.40 M

ployed in this study [39]. Thus, any changes in measured responses as a function of pH must be attributed to secondary solute–stationary phase effects.

Factor–factor interactions, which are typically ignored in many experimental designs, occur in most chemical systems [36,40,41]. Generally, a first-order model incorporating interaction effects between factors may be described by:

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

In this study, x_1 is the pH, x_2 is the concentration of methanol ([CH_3OH]), x_3 is the ionic strength (I), the b_i's are regression coefficients and r is the response. Responses considered for the model are partition coefficient (K_{sec}) and number of theoretical plates (N). Volumetric flowrates, measured by weighing portions of the eluent after specific time intervals, are constant to within 1% of the pump setting. Hence, K_{sec} is calculated with retention times rather than elution volumes according to:

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

where t_e, t_o and t_t are the elution times of the analyte, blue dextran and the nitrate anion, respectively. The values of N are calculated 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.

Overall K_{sec} ($K_{sec,sum}$) and 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 N values of 15 000 and 300 and area percentages of 90% and 10%, respectively, a calculated N_{sum} of 13 530 would result ($= 0.9 \cdot 15\,000 + 0.1 \cdot 300$). A greater weight in the overall response is therefore given to the peak with the higher relative abundance. Since SEC peaks are generally wide with low values of N, area measurements are felt to give greater accuracy than height when calculating relative abundances.

A FED calculation yields 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 [31,33,34]. Since a quantitative model is not being developed here, a qualitative approach will be employed. In this study the qualitative data matrix may be represented by [D]:

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

in which the first, second and third columns list pH, [CH_3OH] and I, respectively, and where low values for each factor are represented by -1 and high values by 1. The corresponding quan-

titative values may be found in Table 1. This method of coding is analogous to normalization in that bias due to differences in the magnitudes of individual factors reported in diverse units is eliminated [42]. In this case where solute–stationary phase interactions in SEC are examined, a qualitative model allows the determination of which interactions are present under the experimental conditions [31,36]. Also, a measure of the relative importance of each interaction to the calculated responses is possible.

The corresponding qualitative regression matrix, derived from Equation 1, is defined as

$$[X] = \begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & -1 & 1 & -1 & -1 & -1 \\ 1 & -1 & 1 & -1 & 1 & -1 & -1 \\ 1 & -1 & -1 & -1 & -1 & 1 & 1 \\ -1 & 1 & 1 & -1 & -1 & 1 & -1 \\ -1 & 1 & -1 & -1 & 1 & -1 & 1 \\ -1 & -1 & 1 & 1 & -1 & -1 & 1 \\ -1 & -1 & -1 & 1 & 1 & 1 & -1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & -1 & 1 & -1 & -1 & -1 \\ 1 & -1 & 1 & -1 & 1 & -1 & -1 \\ 1 & -1 & -1 & -1 & -1 & 1 & 1 \\ -1 & 1 & 1 & -1 & -1 & 1 & -1 \\ -1 & 1 & -1 & -1 & 1 & -1 & 1 \\ -1 & -1 & 1 & 1 & -1 & -1 & 1 \\ -1 & -1 & -1 & 1 & 1 & 1 & -1 \end{bmatrix}$$

in which the columns from left to right represent x_1, x_2, x_3, x_1x_2, x_1x_3, x_2x_3 and $x_1x_2x_3$. The regression coefficients b_1, b_2 and b_3 describe the effects of the main factors pH, [CH_3OH] and I respectively. The regression coefficients b_{12}, b_{13} and b_{23} represent the binary pH–[CH_3OH], pH–*I* and [CH_3OH]–*I* interactions, respectively. The b_{123} coefficient accounts for tertiary interactions between the three main factors, pH–[CH_3OH]–*I*. Interpretation of the coefficients in terms of solute–stationary phase interactions is subjected to bias due to the differing magnitudes of the calculated responses ($K_{sec,sum}$ and N_{sum}). The response data are therefore normalized by multiplying each measured response by a normalization constant [42], N_c $[(\sum r_i)^{-0.5}]$. A different normalization constant is computed for each set of responses derived for each solute on the two stationary phases. Thus, in this study 12 different normalization constants were calculated. Normalized responses (*i.e.*, $K_{sec,sum}$ and N_{sum}) are in the range $0 \geq r \geq 1$.

Parameters of Eq. (1) determined by the LINEST function of Microsoft EXCEL 4.0 include: individual b_i coefficients, error values for each coefficient, *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 regression coefficient is determined by comparing the ratio of the regression coefficient to its respective error value with the critical value of $t_{crit} = 1.86$ of the one-sided *t*-test at the 95% confidence level with v_2 degrees of freedom ($v_2 = n - (k + 1)$, where n = number of experiments = 16, k = number of variables in the regression analysis = 7). Calculated ratios smaller than the critical value are not considered significant and are omitted from Tables 2 and 3. One-sided *F*-tests are applied to regression equations to determine the significance of the proposed model [as expressed by Eq. (1)] using a 95% confidence level 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 $F_{crit} = 3.73$ are considered insignificant. This would imply that the measured responses are not adequately described by the proposed model.

3.1. $K_{sec,sum}$

Example chromatograms for collagen, Croda glue and VM gelatin on the silica stationary phase are shown in Figs. 1–3, respectively. Similarly, chromatograms for collagen, Croda glue and VM gelatin on the polymeric stationary phase are shown in Figs. 4–6, respectively.

Calculated regression coefficients of $K_{sec,sum}$ for collagen, Croda glue and VM gelatin are shown in Table 2. The factors or factor–factor interactions corresponding to each b_i coefficient are shown in the first row. The polymeric phase

Table 2
Calculated regression coefficients for $K_{sec,sum}$

Sample	Column	F_{calc}	b_0	b_1 pH	b_2 CH_3OH	b_3 I	b_{12} pH–CH_3OH	b_{13} pH–I	b_{23} CH_3OH–I	b_{123} pH–CH_3OH–I
Collagen	TSKGel	1974	0.225	−0.029	−0.011	0.097	−0.015	−0.015	−0.026	−0.015
	BioSi	267	0.202	−0.071	0.048	0.060	−0.020	−0.035	0.025	−0.014
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	−0.035	−0.006	0.014	−0.001
VM	TSKGel	87	0.247	−0.010	0.007	0.022	0.005		−0.025	−0.003
	BioSil	69	0.249	0.007	0.019	0.014				

Table 3
Calculated regression coefficients for N_{sum}

Sample	Column	F_{calc}	b_0	b_1 pH	b_2 CH_3OH	b_3 I	b_{12} pH–CH_3OH	b_{13} pH–I	b_{23} CH_3OH–I	b_{123} pH–CH_3OH–I
Collagen	TSKGel	4673	0.160	−0.056	0.075	0.035	−0.062	−0.073	0.108	−0.078
	BioSil	67	0.243	−0.021	−0.031	−0.015	−0.006	0.034	0.031	0.024
Croda	TSKGel	3916	0.197	−0.055	0.018	−0.065	−0.057	−0.007	0.114	−0.005
	BioSil	133	0.233	−0.044	−0.043	−0.062	−0.021	0.010	−0.008	0.010
VM	TSKGel	1087	0.222	−0.085	−0.010		0.021	−0.036	0.056	0.033
	BioSil	212	0.220	−0.068	−0.073	−0.044	0.008		0.044	−0.017

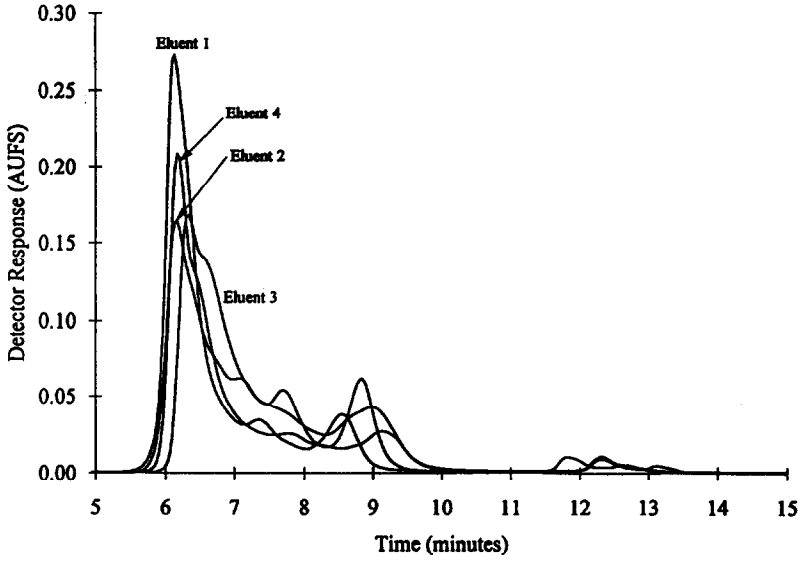

Fig. 1. Example chromatograms for the elution of collagen from the BioSil SEC-250 silica-based stationary phase. Eluent 1: pH = 7, [CH_3OH] = 0%, I = 0.1 M; Eluent 2: pH = 4, [CH_3OH] = 0%, I = 0.1 M; Eluent 3: pH = 7, [CH_3OH] = 15%, I = 0.1 M; Eluent 4: pH = 7, [CH_3OH] = 0%, I = 0.4 M.

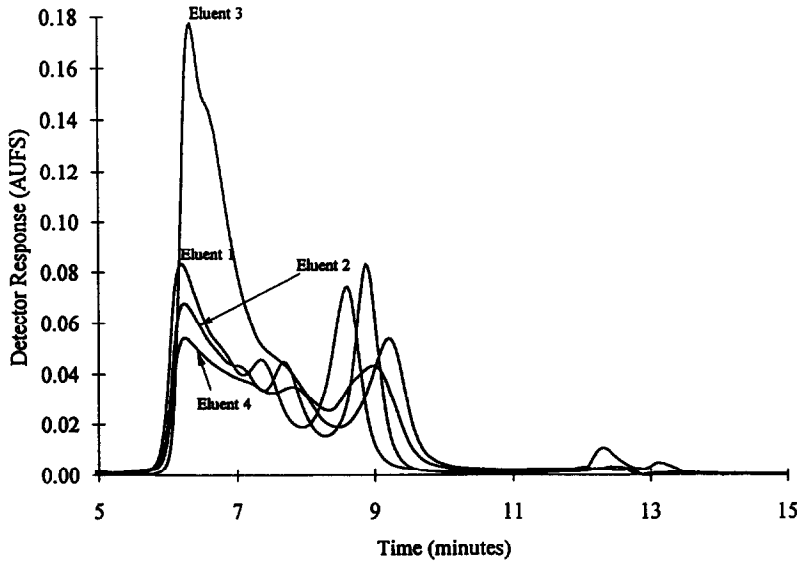

Fig. 2. Example chromatograms for the elution of Croda Glue from the BioSil SEC-250 silica-based stationary phase. Eluent 1: pH = 7, $[CH_3OH] = 0\%$, $I = 0.1$ M; Eluent 2: pH = 4, $[CH_3OH] = 0\%$, $I = 0.1$ M; Eluent 3: pH = 7, $[CH_3OH] = 15\%$, $I = 0.1$ M; Eluent 4: pH = 7, $[CH_3OH] = 0\%$, $I = 0.4$ M.

is represented by TSKGel and the derivatized silica phase by BioSil. Calculated F statistics (F_{calc}) greater then F_{crit} ($= 3.73$) imply 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.

Elution times for nitrate exhibit relative stan-

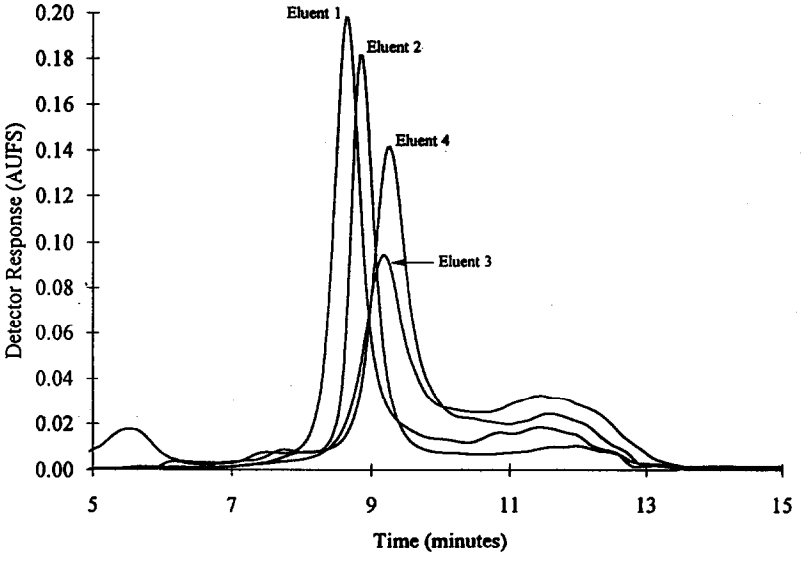

Fig. 3. Example chromatograms for the elution of Vieille-Montagne gelatin from the BioSil SEC-250 silica-based stationary phase. Eluent 1: pH = 7, $[CH_3OH] = 0\%$, $I = 0.1$ M; Eluent 2: pH = 4, $[CH_3OH] = 0\%$, $I = 0.1$ M; Eluent 3: pH = 7, $[CH_3OH] = 15\%$, $I = 0.1$ M; Eluent 4: pH = 7, $[CH_3OH] = 0\%$, $I = 0.4$ M.

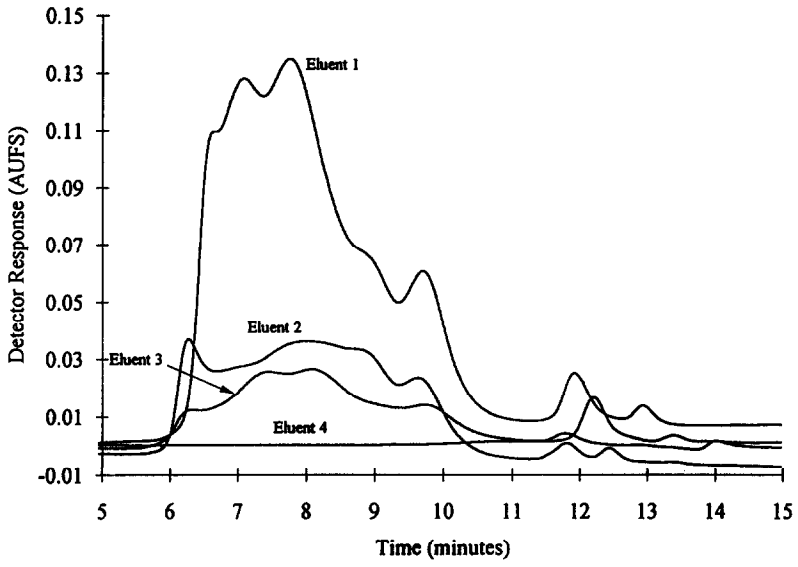

Fig. 4. Example chromatograms for the elution of collagen from the TSK Gel 4000PW$_{xL}$ polymeric stationary phase. Eluent 1: pH = 7, [CH_3OH] = 0%, I = 0.1 M; Eluent 2: pH = 4, [CH_3OH] = 0%, I = 0.1 M; Eluent 3: pH = 7, [CH_3OH] = 15%, I = 0.1 M; Eluent 4: pH = 7, [CH_3OH] = 0%, I = 0.4 M.

dard deviations (R.S.D.) of 2.9% and 2.6% on the polymeric and derivatized silica phases, respectively for the 16 different experiments using 8 distinct eluents. These 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 ion interaction effect for the

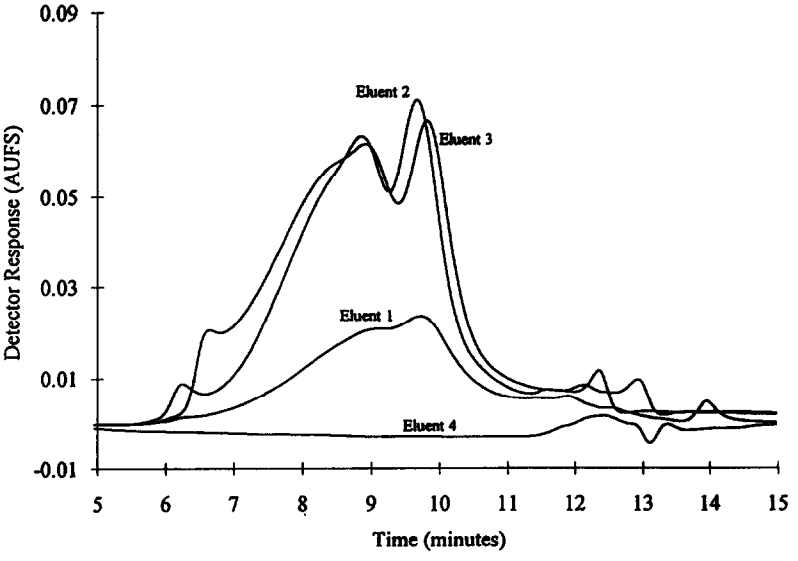

Fig. 5. Example chromatograms for the elution of Croda Glue from the TSK Gel 4000PW$_{xL}$ polymeric stationary phase. Eluent 1: pH = 7, [CH_3OH] = 0%, I = 0.1 M; Eluent 2: pH = 4, [CH_3OH] = 0%, I = 0.1 M; Eluent 3: pH = 7, [CH_3OH] = 15%, I = 0.1 M; Eluent 4: pH = 7, [CH_3OH] = 0%, I = 0.4 M.

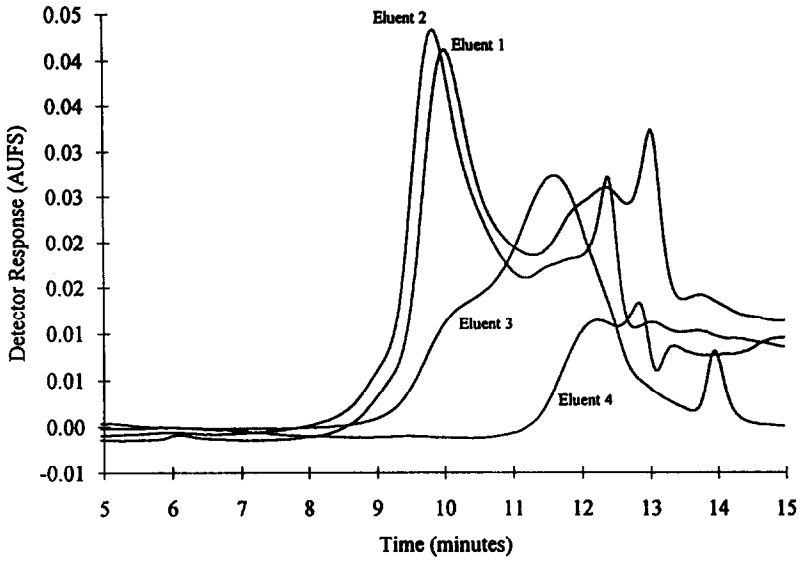

Fig. 6. Example chromatograms for the elution of Vieille-Montagne gelatin from the TSK Gel 4000PW$_{xL}$ polymeric stationary phase. Eluent 1: pH = 7, [CH_3OH] = 0%, I = 0.1 M; Eluent 2: pH = 4, [CH_3OH] = 0%, I = 0.1 M; Eluent 3: pH = 7, [CH_3OH] = 15%, I = 0.1 M; Eluent 4: pH = 7, [CH_3OH] = 0%, I = 0.4 M.

NO_3^- anion is not surprising considering that previous work has shown that under conditions where ion exclusion is normally present (pH = 7 and I = 0.1 M) the citrate anion yields a K_{sec} of 0.9 on a silica-based stationary phase of similar composition to that employed in this study [43]. This suggests that this stationary phase does not promote excessive ion exclusion. Also, the above R.S.D. for NO_3^- is calculated over pH and I ranges where ion-exclusion effects are reduced or possibly eliminated. Thus, any small amount of ionic interaction between NO_3^- and the stationary phase that may be present is effectively obscured.

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 would represent a good estimate for all silanols. Therefore, at pH ≥ 7 the surface of the derivatized silica phase will exhibit a net negative charge due to deprotonation of residual, non-derivatized, hydroxyl groups. Similarly, deprotonation of carboxyl groups on the polymeric stationary phase will lead to a net negative charge on this phase at pH ≥ 7. The protein–SDS complexes possess a net negative charge at pH ≥ 7 [39]. The model, represented by Eq. (1), contains a b_1 term to account for the individual effect of pH. Negative values are observed in Table 2 with both columns for most solutes investigated, suggesting ion-exclusion effects are present. The magnitudes of the b_1 coefficients for collagen and Croda glue on the silica-based column (BioSil) are all larger than those for the polymeric column (TSKGel). This indicates a greater influence of pH on the separation process on the silica-based stationary phase and suggests a greater extent of ion exclusion at pH ≥ 7. The lack of a negative effect for VM gelatin on the silica column indicates the lack of ion-exclusion effects suggesting that the SDS–VM gelatin complex may not possess a net negative charge.

Organic modifiers such as CH_3OH typically reduce hydrophobic adsorption of protein solutes onto the stationary phase, effectively reducing their retention times. In the context of this study, a reduced $K_{sec,sum}$ would result leading to negative b_2 coefficients in the linear model. The negative b_2 coefficients observed for collagen and Croda glue on the polymeric phase can be attributed to hydrophobic adsorption which is obvious in Figs. 4 and 5. Hydrophobic adsorption is not observed on the silica-based stationary

phase as evidenced by all positive b_2 coefficients. A plausible explanation for these positive b_2 coefficients may be the hydrophobic adsorption of SDS from the eluent onto the silica stationary phase [44]. The resulting repulsion of the anionic protein–SDS complex with the adsorbed anionic SDS would yield decreased retention times. Desorption of SDS from the stationary phase with methanol would eliminate these secondary repulsion effects and increase $K_{sec,sum}$, leading to positive b_2 coefficients. Although the polymeric phase also adsorbs SDS, hydrophobic adsorption of the protein–SDS complexes probably dominates.

Ion-exclusion effects are typically countered by increasing the ionic strength of the eluent. The critical micelle concentration of SDS also decreases, from approximately 8 m*M* to 0.5 m*M* when the ionic strength changes from 0.0 *M* to 0.4 *M* [45]. However, the binding ratio of SDS to collagen remains stable at 1.4 g SDS/g protein [27]. Stationary phases manifesting ion-exclusion effects are expected to show an increase in $K_{sec,sum}$ with an increase in ionic strength. This is also due to a diminution of the thickness of the electric double layer at the surface of the protein–SDS complexes at high ionic strength [18,23]. A decrease in the thickness of this double layer reduces the effective size of the molecule yielding larger values of K_{sec}. The positive b_3 terms in the linear model for all solutes on both stationary phases do imply that ion-exclusion effects are counteracted and hence long retention times are possible with eluents of high ionic strength.

3.2. N_{sum}

Column efficiency in chromatography, N, is dictated by numerous factors, including particle size of the stationary phase, mass transfer effects and diffusion effects. The diffusion coefficient, D_p, of the biopolymer solute within the pore network of the stationary phase is directly proportional to N [9,46–48] and is in turn determined by the Stokes radius of the solute and by secondary retention effects. For large molecular mass solutes the diffusion coefficient will be small leading to reduced values of N. Where ion exclusion occurs, the diffusion coefficient is also reduced since the solutes are partially or fully excluded from the pore network.

Calculated regression coefficients of N_{sum} for collagen, Croda glue and VM gelatin on the two stationary phases are summarized in Table 3. Negative b_1 coefficients are observed for all solutes on both stationary phases indicating a decrease in N_{sum} with increased pH. Under conditions where ion exclusion is present, protein solutes may be either partially or totally excluded from the pores of the stationary phase due to ionic repulsion between the protein solute and the stationary phase. This leads to a greater resistance to diffusion of the polyionic protein–SDS solutes through the pores of the stationary phase [49]

Similarly b_2 coefficients are positive for collagen and Croda glue on the polymeric phase. All protein–SDS solutes show negative b_2 coefficients on the silica phase. Desorption of hydrophobically adsorbed protein–SDS solutes by CH_3OH is obviously responsible for the negative coefficients. The extent of desorption is readily seen in Figs. 4–6. VM gelatin also appears to adsorb hydrophobically to the polymeric phase although the b_2 coefficient is positive. This may be due to a mathematical effect whereby the peaks obtained under hydrophobic adsorption conditions have smaller widths at similar retention times than non-adsorbed peaks thus yielding higher values of N_{sum}. Hydrophobic adsorption of the protein–SDS complexes onto the silica-based stationary phase was not apparent. Adsorption of SDS from the eluent onto the silica stationary phase was suggested from data for $K_{sec,sum}$. However, Fig. 3 shows a large shift toward the void volume at high [CH_3OH] which is opposite to that expected if SDS was adsorbed. A physical change in the geometry of the protein–SDS complexes due to methanol in the eluent may 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

[46,50]. Consequently the negative b_2 coefficients for N_{sum} on the silica phase are presumably a result of an expansion of the protein–SDS complexes induced by methanol in the eluent.

With the exception of collagen and VM gelatin on the polymeric stationary phase, calculated b_3 terms are negative for all solutes. Hydrophobic adsorption is obviously responsible for the negative b_3 coefficient for Croda glue and VM gelatin on the polymeric stationary phase. Ion exclusion is probably responsible for the negative b_3 coefficients observed for the silica-based stationary phase.

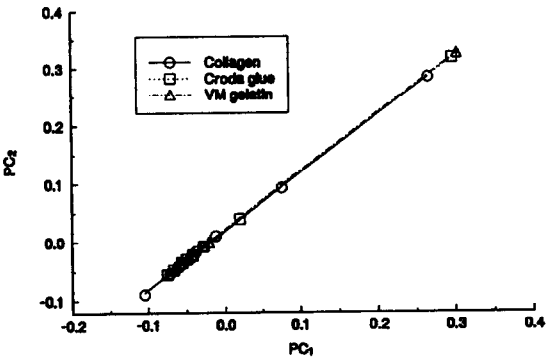

Fig. 7. Principal components of $K_{sec,sum}$.

3.3. Factor–factor interactions

Rather than attempting to rationalize the nature and origin of each factor–factor interaction coefficient for the three responses, an examination of all 8 coefficients through principal component analysis (PCA) may prove more informative. PCA is typically used to reduce the number of features in a large data set to a smaller number of dimensions usually 1-d or 2-d. However, for 2-d data sets where a poor correlation exists, PCA is also useful to clarify the structure of the data [41]. Correlations between two data sets are consequently more precise and easily detected. The detailed theory of PCA can be found elsewhere [42] and will not be discussed here. Equations to calculate 2-d principal components (PC's) [41] may be applied to the FED data with the data vectors represented by calculated regression coefficients for the polymeric and silica stationary phases respectively.

A summary of the linear regression results for the subsequent PC's is shown in Table 4. Excellent linear fits to the data are achieved by the PC's of all protein–SDS solutes for the response $K_{sec,sum}$. Excellent linear fits for collagen and Croda glue are also displayed for N_{sum} while VM gelatin shows a poor fit to the PC data. Principal components for $K_{sec,sum}$ are represented graphically in Fig. 7. Previous interpretation of the main factor effects, separately, implied that collagen and Croda glue behave differently on the two stationary phases when considering $K_{sec,sum}$. Also, VM gelatin did not exhibit ion-exclusion effects. The plot of the PC's for $K_{sec,sum}$ imply that the influence of the main factors and the factor–factor interactions is identical [40] for the three solutes studied. The binary and tertiary interaction coefficients, b_{12}, b_{13}, b_{23} and b_{123}, thus exert appreciable influence on the determination of $K_{sec,sum}$. In fact, the effects of the main factors on $K_{sec,sum}$ appear to be compensated by their interactions.

Similar behaviour of collagen and Croda glue

Table 4
Regression statistics for the principal components of the responses for the TSKGel and BioSil stationary phases

Response	Solute	Slope	Intercept	Variance
$K_{sec,sum}$	Collagen	0.9967	0.0178	0.9996
	Croda Glue	0.9996	0.0192	1.0000
	VM Gelatin	0.9996	0.0212	1.0000
N_{sum}	Collagen	0.9951	0.009	0.9971
	Croda Glue	0.9439	0.0167	0.9043
	VM Gelatin	0.6780	0.0049	0.6040

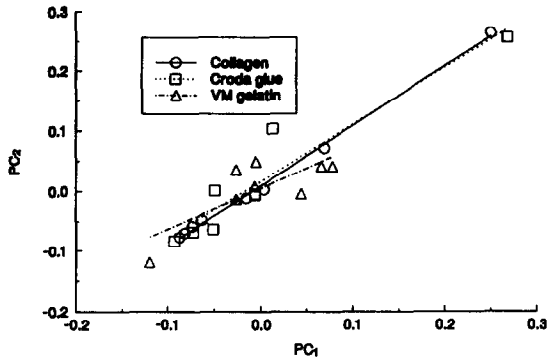

Fig. 8. Principal components of N_{sum}.

recurs for the PC's of N_{sum} as illustrated in Fig. 8. The different slope for the PC's of VM gelatin when compared to those of the other two proteins signifies dissimilarities in the influence of the experimental variables on the separation mechanism. Dissimilarities in the PC's are characteristic of contrasting modes of separation. This further suggests that the diffusion character, and the character of the VM–SDS complex, is distinct from the other two proteins studied.

4. Conclusions

The FED approach affords a rapid method to determine qualitatively the effects of various factors on the SEC separation of known and unknown biopolymers with different stationary phases. Contributions to non-ideal separation effects are readily seen through regression analysis of the FED data. When examined separately, main factor coefficients imply the existence of significant ion-exclusion effects on both the polymeric and derivatized silica phases employed in this study. The silica-based stationary phase exhibits a greater proclivity towards ionic exclusion than the polymeric stationary phase. This is due in part to a greater number of hydroxyl groups which are subject to deprotonation at pH $\geq$ 7. Methanol apparently induces an increase in the Stokes radius of protein–SDS complexes as seen by the b_2 coefficients for N_{sum}. Also, adsorption of SDS from the eluent onto the silica stationary phase, which was evident by the b_3 coefficients for $K_{sec,sum}$, supplements existing ion exclusion by the silanol groups on the silica stationary phase. Hydrophobic adsorption of the collagen–SDS and Croda glue–SDS solutes on the polymeric column was also revealed by the b_3 regression coefficients. The presence of hydrophobic adsorption of the hydrophilic protein–SDS complexes indicates a high degree of hydrophobicity of the polymeric stationary phase.

Coefficients derived from the FED suggest that maximum values of N_{sum} can be achieved with an eluent at pH = 7, $[CH_3OH] = 0\%$ and $I = 0.1$ M for collagen and Croda glue on the polymeric stationary phase. A pH of 4 is required to yield optimum results for VM gelatin on the same phase. All protein solutes show optimum values of N_{sum} at pH = 4, $[CH_3OH] = 0\%$ and $I = 0.1$ M on the silica-based stationary phase. These eluent conditions do not eliminate secondary, non-ideal SEC effects but they do provide maximum separation efficiency as defined by N_{sum}.

VM gelatin behaves in an abnormal fashion when compared to collagen and Croda glue as evident by dissimilar main factor coefficients derived from the FED. Ionic strength and CH_3OH change the effective molecular size of collagen and Croda glue but do not appear to alter VM gelatin. This abnormal behaviour may be correlated to a difference in the structure of the VM–SDS complex when compared to those of collagen and Croda glue.

Principal component analysis of all main factor coefficients and factor–factor interaction coefficients for the two responses reflects the importance of the factor–factor interactions which are typically ignored when designing chromatographic separation strategies. The effect of the three experimental factors and their interactions on $K_{sec,sum}$ and hence the apparent molecular mass is identical for the three species examined on both columns. Diverging PC's for VM for N_{sum} imply distinct separation mechanisms and hence a different structure of the protein–SDS complex when compared to both collagen and Croda glue. The type I collagen used as a control sample is of similar reported composition to Croda glue and VM gelatin. The PC's for VM

gelatin and in fact the individual main factor coefficients contradict this premise. We may conclude that the VM gelatin is not composed of the same collagen fragments which make up the Type I collagen and the Croda glue employed in this study.

Overall, the combination of FED and PCA is a useful tool for revealing the nature and extent of solute–stationary phase interactions in SEC separations. When dealing with unknown biopolymers, such as Croda glue and VM gelatin, an understanding of these secondary effects is critical for obtaining valid information on the properties of these molecules through SEC. The significance of binary and tertiary factor–factor interactions cannot be over emphasized. Most experimental designs employ the typical single-factor-at-a-time strategy whereby the crucial interactions of individual factors are ignored. This study has demonstrated, through FED and PCA, the importance of binary and tertiary factor interactions for the interpretation of parameters characteristic of SEC.

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