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Characterization of the influence of displacing salts on retention in gradient elution ion-exchange chromatography of proteins and peptides

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ABSTRACT

It has been shown earlier that the choice of displacing salt has a large effect on the retention in ion-exchange chromatography of proteins and peptides. The influence of different displacing salts cannot be predicted or quantitatively explained, owing to the current lack of an adequate theoretical framework. In this work a general characterization is made by using a considerable number of proteins and peptides and all displacing salts found feasible. Principal component analysis is used to interpret the large amount of data that is generated. The results of the analysis indicate that most of the retention variations are due to non-specific effects and can be explained by changes in the apparent gradient slope, *i.e.*, the increase in elution strength per unit volume, and the elution strength of the starting buffer. This differs from the interpretation given earlier, where the selectivity changes were attributed to specific effects of the salts. However, as it is impossible to test all existing proteins and peptides, specific effects are still possible, but they might be less common than previously considered.

INTRODUCTION

Ion-exchange chromatography (IEC) is a major separation technique for proteins and peptides. Maintaining the biological activity is often crucial for these substances, and ion-exchange chromatography allows the use of mild separation conditions. Further, the load capacity is high and most proteins and peptides can be "trapped" on an ion-exchange column, making it a suitable technique for trace enrichment and preparative separations.

The separation of proteins and peptides by IEC is usually made with a salt gradient, going from a pure buffer solution to a solution of salt and buffer. The concept of *elution strength* is essential in the following discussion and will therefore be presented in some detail. The elution strength of an eluent is the ability to elute solutes from the column. For a given stationary phase, the elution strength is determined by the concentration and type of ionic species in the eluent. The elution strength is therefore lower in the starting buffer than in the eluting buffer. This leads to an increase in elution strength during the gradient. The rate of increase in elution strength per unit volume will in this paper be referred to as the *apparent gradient slope*. The apparent gradient slope can, for a given salt, be altered by changing the salt concentration of the eluting buffer, the flow-rate or the gradient time. It is important to realise, however, that the apparent gradient slope will generally be different if the displacing salt is changed, even if these factors are kept constant. This will be further discussed later in this paper.

The selectivity in IEC can be altered in several ways, but the most obvious parameter to adjust is the pH of the buffer as it directly affects the charge of the solutes. In some instances the available pH range is restricted owing to properties of the sam-

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ple, *e.g.*, solubility or stability. The limited pH range may permit only minor changes in the selectivity, which might not be sufficient for adequate resolution. Previous studies indicate that the choice of displacing salt has a significant impact on the separation [1]. The effect can be non-specific, *i.e.*, just a change in the apparent gradient slope, changing the retention of all solutes in a similar manner. Specific effects, on the other hand, influence individual solutes differently. The conclusions drawn in earlier studies are that both retention and selectivity depend on the type of displacing salt [2]. In addition, it has been observed that in both anion-exchange chromatography (AEC) and cation-exchange chromatography (CEC), the displacing counter-ion and the co-ion are of importance [2–4].

Unfortunately, there is at present no theory that quantitatively explains the effect of changing the displacing salt [2,5]. Studies of various displacing salts therefore tend to be empirical and the results can rarely be generalized.

The aim of this work was to describe and evaluate the effect of various displacing salts. In the absence of a valid theory, an empirical characterization is made by utilizing a multivariate method. This approach is analogous to the method used by Coenegracht *et al.* [6] for the characterization of solvent strength and selectivity in isocratic reversed-phase liquid chromatography. The study presented here includes a considerable number of proteins and peptides and evaluation of all salts found feasible, to make a general characterization. All possible combinations of the selected cations and anions were taken into consideration when establishing the list of suitable salts. The proposed approach should be contrasted with previous studies where only salts having either the cation or the anion in common, *e.g.*, various sodium salts and chlorides, were used [2,3].

Parts of the data presented in this paper have been analysed previously and presented with a different interpretation [7].

METHOD FOR DATA ANALYSIS

The primary outcome of the experiments in this study is a large amount of results, expressed as tables of retention data for proteins and peptides eluted with various salts. There is an obvious need for data reduction and graphical illustration, as the tables are difficult to overview and interpret. Chemometrics provides a tool for this called *principal component analysis (PCA)*, which is an efficient method for analysing and illustrating such data. The results can be presented as plots, illustrating the influence of various displacing salts in a way that enhances and simplifies the interpretation. The fundamental concept of PCA is that the variations observed in a large number of variables essentially is caused by variations in a few underlying properties. The number of underlying sources of variation is usually far less than the number of observed variables.

In this paper, PCA will be explained geometrically and for this distinct application only. The reader is referred to tutorials or chemometrics textbooks for a more complete description [8–10]. For instructive purposes, we shall first assume that only three solutes were used in this study. Elution with one salt will then result in three retention volumes, associated with this particular salt. The salt can be represented as a point in a three-dimensional (three solutes) space. All the other salts can be represented in the same way, resulting in a number of points, as illustrated in Fig. 1. Two salts that give about the same retention volumes will lie close to each other in this space, and a large difference in retention implies a large distance.

Three-dimensional spaces are difficult to illustrate and interpret. The three dimensions are therefore reduced by PCA to a two-dimensional space according to the following procedure. First, a vector, called a *principal component*, is drawn in the direction showing the greatest spread among the points. Second, another principal component, perpendicular to the first, is drawn in the direction were most of the remaining spread is found. This is illustrated in Fig. 2. These two components (designated PC1 and PC2) now define a plane, on which all points can be projected (Fig. 3). This projection expresses as much of the original spread as possible in only two dimensions, making the presentation and interpretation easier. The data reduction described above can be done from any number of dimensions, *i.e.*, any number of *variables*. In this context a variable corresponds to the retention volume for one solute.

There are some important terms in PCA that need to be described in more detail to facilitate the

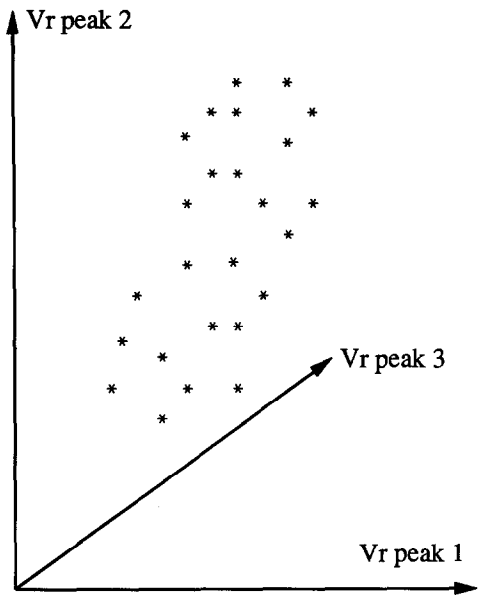

Fig. 1. Retention volumes for three solutes plotted in a three-dimensional coordinate system. The salts (objects) are characterized by the retention volumes (variables).

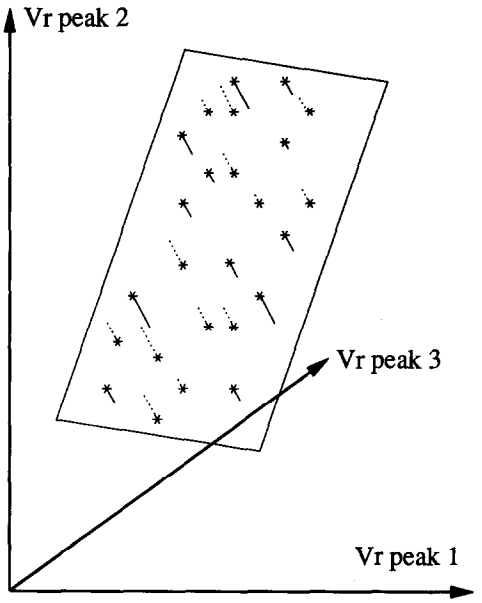

Fig. 3. All objects projected on the plane defined by the two first principal components.

following discussion. Each salt, generally denoted an *object*, is characterized by several retention volumes, *i.e.*, values for the variables. The salts will acquire new values when they are projected on the plane defined by the principal components as illustrated in Fig. 3. These values in the coordinate system constituted of the principal components are referred to as *scores*. The number of principal components that is needed to express the variation, excluding noise or small experimental errors, in the original data is the *rank* of the data. The rank can be determined by several methods, but the most commonly used is cross-validation [8]. In many cases, two or three components are enough to represent the main part of the original spread. Each principal component is a linear combination of the original variables. Depending on the direction of a component in the space it will express a different combination of the original variables. How much of an original variable that goes into a specific component is given by the so-called *loading* of the variable.

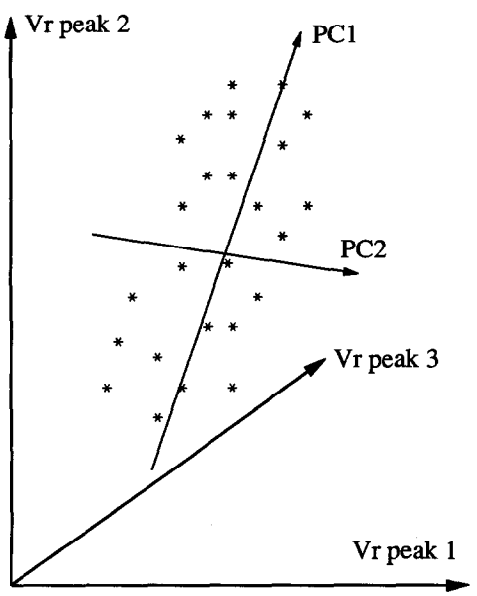

Fig. 2. The two first principal components.

A *score plot* can be produced where the salts are spread out in the plane defined by the principal components showing their general influence on the retention. A *loading plot* shows the mix of the individual solute retentions that is constituting each principal component. When interpreting the plots it is important to realize that although the number of significant principal components is equivalent to

the number of underlying factors, a principal component is not equivalent to an underlying factor. Principal components are always orthogonal, which is not necessarily true for the underlying factors. The underlying factors are combinations of the principal components and vice versa.

Usually, the data are manipulated prior to the determination of the principal components. The variables are often *weighted* to make them more equal in variation. This has not been done in this work as all data have similar experimental errors and are directly comparable.

EXPERIMENTAL

Instrumentation and software

The chromatographic experiments were performed at ambient temperature (22–25°C) using an FPLC system (Pharmacia LKB Biotechnology, Uppsala, Sweden) consisting of two P-500 pumps, MV-7 and MV-8 valves controlled by an LCC-500 liquid chromatography controller. The injections were made with a CMA 200/240 refrigerated (4°C) autosampler (CMA Microdialysis, Stockholm, Sweden) controlled by a Toshiba T-1000 laptop computer. Detection at 214 and 280 nm, for CEC and AEC, respectively, was done with a Linear model 200 (Linear Instruments, Reno, NV, USA) variable-wavelength UV detector coupled to a Spectra-Physics (San Jose, CA, USA) SP4290 integrator. For the proteins a strong anion-exchange column (Mono Q HR 5/5; Pharmacia LKB Biotechnology) was used, while a strong cation-exchange column (Mono S HR 5/5, Pharmacia LKB Biotechnology) was used for the peptides. The pH measurements were carried out with a Metrohm (Herisau, Switzerland) Model 654 pH meter equipped with a combination glass electrode.

The raw data from the integrator were transferred to an IBM PS/2 computer for determination of retention volumes by in-house developed software. The principal component analyses were performed on the IBM PS/2 computer, using the program UNSCRAMBLER (Camo, Trondheim, Norway).

Chemicals and reagents

Horse muscle myoglobin, bovine erythrocyte carbonic anhydrase, human transferrin, bovine serum albumin and soybean trypsin inhibitor were all purchased from Sigma (St. Louis, MO, USA). Calf intestine alkaline phosphatase and *Aspergillus Niger* amyloglucosidase were both purchased from Fluka (Buchs, Switzerland). Human immunoglobulin G and all peptides were provided by Pharmacia LKB Biotechnology.

The buffers were prepared from tris(hydroxymethyl)aminomethane (Tris) (Merck, Darmstadt, Germany), piperazine (Sigma) and phosphoric acid (Merck), all of analytical-reagent grade. Purified water was obtained from a Milli-Q Plus system (Millipore, Bedford, MA, USA) equipped with an Organex-Q cartridge to remove organic contaminants. Acetonitrile was of gradient grade from Merck.

The acids used for pH adjustment of the buffers in AEC were hydrochloric, acetic, hydrobromic, formic, tartaric, citric (May & Baker, Dagenham, UK), phosphoric and sulphuric acid, all of analytical-reagent grade from Merck except for citric acid. For CEC the pH adjustments were done with potassium hydroxide (J.T. Baker, Deventer, Netherlands), sodium hydroxide (Eka Nobel, Surte, Sweden), lithium hydroxide (Riedel-de Haën, Seelze-Hannover, Germany) or ammonia (Merck), all of analytical-reagent grade.

The salts used were all of analytical-reagent grade and the amount of crystal water is indicated in parentheses where appropriate: ammonium acetate, ammonium chloride, ammonium dihydrogenphosphate, ammonium sulphate, calcium chloride(2), lithium chloride, magnesium acetate(4), magnesium chloride(6), magnesium sulphate(7), potassium bromide, potassium chloride, sodium acetate, sodium chloride, sodium formate, sodium dihydrogenphosphate(1), sodium sulphate and sodium tartrate(2) were purchased from Merck, ammonium bromide, ammonium formate, lithium acetate and potassium citrate(1) from BDH (Poole, UK), calcium bromide, lithium citrate(4) and sodium perchlorate from Fluka, lithium bromide, potassium formate and potassium tartrate from Sigma, lithium sulphate(1), potassium sulphate and potassium dihydrogenphosphate from J.T. Baker, calcium acetate (1) and sodium citrate(2) from Fisher (Fair Lawn, NJ, USA), lithium perchlorate from Janssen Chimica (Beerse, Belgium) and sodium bromide from Baker & Adamson (Allied Chemicals, New York, NY, USA).

Chromatographic procedure

For AEC of proteins at pH 8.0, the starting buffer was 10 m*m* Tris and the eluting buffer included 10 m*m* Tris and the displacing salt with a molality corresponding to a displacing ionic strength, defined later in this paper, of 1 *M*. When the AEC experiments were performed at pH 9.6, 10 millimolal piperazine was instead used as the buffer substance. To avoid uptake of carbon dioxide, the pH 9.6 buffers were kept in closed vessels. The pH of the eluents was in both instances adjusted with the acid corresponding to the displacing anion. For the ammonium salts, the pH of the eluting buffer was adjusted using ammonia. All eluents were filtered through 0.22-μm Millipore-GS filters and degassed by sparging with helium for 5 min. The flow-rate was 1 ml/min and the gradients went linearly from 0 to 98% of the eluting buffer in 28 min, corresponding to an increase of 3.5%/ml. Various amounts between 0.3 and 1.3 mg/ml of the protein samples were individually dissolved in the starting buffer at the appropriate pH adjusted with acetic acid. The protein samples were filtered through a 0.45-μm Millex-HV filter (Millipore) before injection of 50 μl of each protein. Prior to the injection, the solubility of each protein in the eluting buffers was tested. The proteins were dissolved in all eluting buffers and incubated at 4°C for 12 h. The presence of an insoluble pellet was examined after centrifugation at 3000 *g* for 5 min.

For CEC of peptides the starting buffer was 12 m*m* phosphoric acid and the eluting buffer consisted of 12 m*m* phosphoric acid and the displacing salt corresponding to a displacing ionic strength of 1 *M*. The pH was adjusted to 2.2 with the base corresponding to the displacing cation. For the magnesium and calcium salts, the pH adjustment was instead performed with lithium hydroxide owing to the limited solubility of $Mg(OH)_2$ and $Ca(OH)_2$. The eluting buffers containing sulphate or phosphate were pH adjusted with sulphuric and phosphoric acid, respectively. After the pH adjustment, 30% (v/v) of acetonitrile was added to both eluents. The eluents were filtered and degassed as described above. The gradients went linearly from 0 to 70% of the eluting buffer in 20 min (increase 3.5%/ml). The peptides were individually dissolved in acetonitrile before injection of 0.5–5 μg of each peptide.

RESULTS AND DISCUSSION

Chromatographic measurements

A careful selection of representative proteins and peptides was used in this study. The eight proteins were systematically chosen with various isoelectric points and molecular masses (see Table I). The nine selected peptides also span a wide range of hydrophobicities (see Table II). However, it is of course impossible to represent all natural variations in solute properties by a small number of proteins and peptides.

IEC can be performed as either a cation- or anion-exchange procedure. AEC was chosen for the protein studies, because it is commonly used and many biological proteins are slightly acidic. For peptides, on the other hand, CEC is usually selected as many biologically active peptides are basic. It will be seen in the following discussion that the choice of ion-exchange mode has an impact on which pH is appropriate, and hence also on buffers and possible salts.

The type of buffer substance that should be used is determined by the ion-exchange mode, pH and detection method. Peptides are preferably detected by UV absorbance at 210–230 nm, which excludes many commonly used buffers. If the cation-exchange mode is used, the only possible buffer substance will be phosphate. To ensure retention one has to operate around the lower pK_a of phosphate and all CEC separations in this work were performed at a pH of 2.2. With proteins, detection is done at

TABLE I

SELECTION OF PROTEINS FOR AEC

Abbreviation[a]	Protein	10^3 M_r[b]	p*I*[b]
Myo	Myoglobin	17.5	6.8, 7.2
CA	Carbonic anhydrase	30	5.9
IgG	Immunoglobulin G	160	7.7
Tra	Transferrin	77	6.0–6.5
APh	Alkaline phosphatase	140	4.4
BSA	Bovine serum albumin	67	5.1
STI	Soybean trypsin inhibitor	21.5	4.5
Amy1, Amy2	Amyloglucosidase	97	3.6

[a] Used for identification of protein peaks in figures and tables.
[b] M_r = relative molecular mass and p*I* = isoelectric point. Data from refs. 11 and 12.

TABLE II

PEPTIDES USED IN CEC

Abbreviation[a]	M_r	p*I*	Hydrophobicity[b]	Sequence
p1	589	3.1	13	Met–Val–Asn–Pro–Glu
p2	561	9.9	10	Ser–Val–Pro–Met–Lys
p3	571	3.1	22	Tyr–Glu–Leu–Phe
p4	626	8.4	24	Pro–Leu–Ile–His–Phe
p5	1071	6.5	26	Thr–Pro–Ile–Pro–Arg–Tyr–Pro–Leu–Asp
p6	1858	3.9	15	His–Thr–Asp–Arg–Glu–His–Thr–Ile–Glu–Thr–Asp–Glu–Met–Glu–Asp
p7	1729	9.5	10	Lys–Tyr–Gly–Asn–Leu–Ser–His–Glu–Lys–Gln–His–Gln–Leu–Phe
p8	1689	3.1	55	Gly–Asn–Gly–Gln–Asp–Val–Met–Ala–Leu–Ala–Thr–Ile–Leu–Ser–Trp–Leu
p9	1722	9.6	45	Gln–Leu–Ser–Leu–Ala–Ile–Phe–His–Ser–Thr–Tyr–Trp–Lys–Ala–Gly

[a] Used for identification of peptides in figures and tables.
[b] Calculated according to ref. 13.

a wavelength in the range 250-280 nm, which extends the range of useful buffers compared with the peptide separations. Tris buffer with a pH of 8.0 was chosen in this study, simply because it is a commonly used buffer for AEC. For comparison some salts were also tested at a pH of 9.6 with piperazine as buffer substance.

Hydrophobic interaction is a common problem in the separation of peptides by ion-exchange columns [14–16]. To minimize this effect, 30% (v/v) of acetonitrile was added to the mobile phases used in the peptide separations.

Since it has been shown that the choice of both the displacing counter-ion and the co-ion can affect the retention, all possible combinations of the cations and anions listed in Table III have been considered. All candidate salts had to fulfil several conditions, listed in Table IV, to qualify for evaluation in this work. These conditions are essentially demands that have to be met for a salt to make it useful in practice. Owing to the different chromatographic conditions and detection wavelengths, different selections of salts were made for the peptide and protein separations. The final selection of the salts found feasible is listed in Table V for protein separations and in Table VI for peptides.

Different salts give different elution strengths. If the same concentration of the displacing salt were used in all eluting buffers, large differences in the apparent gradient slope would appear for salts with different valencies of the displacing ion. In order to minimize the influence of the displacing ion valency, the concentration in the eluting buffer must be adjusted. Intuitively, and according to a proposed theory for ion-exchange chromatography [5], the retention is primarily controlled by the concentration and valency of the displacing ion. The concentrations might thus be adjusted proportionally to the valency of the displacing ion, *i.e.*, making the salt concentration half as high for divalent displacing ions compared with monovalent ions. However, preliminary experiments indicated that large differences in the elution strength still remained. The eluting buffers were therefore instead prepared with constant *displacing ionic strength* (I_D) calculated by the equation $I_D = m_D z_D^2$, where m_D and z_D denotes the molality and valency of the displacing ion, re-

TABLE III

ANIONS AND CATIONS CONSIDERED FOR ION EXCHANGE

Cations	Anions
Potassium	Acetate
Lithium	Hydrogen carbonate
Sodium	Bromide
Ammonium	Chloride
Barium	Formate
Calcium	Iodide
Magnesium	Perchlorate
	Phosphate
	Propionate
	Oxalate
	Sulphate
	Tartrate
	Citrate

TABLE IV

CONDITIONS FULFILLED FOR THE SELECTED SALTS

Condition	Remark
Soluble	The salt should be soluble in the solvent and at the pH used
Non-denaturating	Some salts, *e.g.*, heavy metals, are known to denature several proteins
Stable	The salt has to be stable under normal laboratory conditions; it should not be decomposed by light or be extremely hydroscopic
Transparent	The salt should not interfere with the UV detection at 210–230 nm for peptides or 250–280 nm for proteins
Ionic at relevant pH	Many ions are protolytic and exist as ions only in a specific pH range, *e.g.*, acetate
pH stable	The hydrogencarbonates were discarded owing to the risk of carbon dioxide evolution. When trying the perchlorates at pH 8.0, it was difficult to obtain a stable pH value
Non-hazardous	It should not be dangerous or inconvenient to handle the salt
Available	The salt of analytical-reagent grade should be commercially available

TABLE V

SELECTION OF SALTS FOR AEC OF PROTEINS AT pH 8.0

No.[a]	Salt	Molality		Valency		Ionic strength	
		m_{cation}	m_{anion}	z_{cation}	z_{anion}	I_D[b]	I[c]
1	Lithium acetate	1.0	1.0	1+	1−	1	1.0
2[d]	Sodium acetate	1.0	1.0	1+	1−	1	1.0
3	Ammonium acetate	0.99	1.0	1+	1−	1	1.0
4	Calcium acetate	0.50	1.0	2+	1−	1	1.5
5	Magnesium acetate	0.50	1.0	2+	1−	1	1.5
6	Potassium bromide	1.0	1.0	1+	1−	1	1.0
7[d]	Lithium bromide	1.0	1.0	1+	1−	1	1.0
8	Sodium bromide	1.0	1.0	1+	1−	1	1.0
9	Ammonium bromide	0.99	1.0	1+	1−	1	1.0
10[d]	Calcium bromide	0.50	1.0	2+	1−	1	1.5
11	Potassium chloride	1.0	1.0	1+	1−	1	1.0
12[d]	Lithium chloride	1.0	1.0	1+	1−	1	1.0
13[d]	Sodium chloride	1.0	1.0	1+	1−	1	1.0
14	Ammonium chloride	0.99	1.0	1+	1−	1	1.0
15	Calcium chloride	0.50	1.0	2+	1−	1	1.5
16	Magnesium chloride	0.50	1.0	2+	1−	1	1.5
17[d]	Potassium formate	1.0	1.0	1+	1−	1	1.0
18	Sodium formate	1.0	1.0	1+	1−	1	1.0
19	Ammonium formate	0.99	1.0	1+	1−	1	1.0
20[d]	Potassium sulphate	0.50	0.25	1+	2−	1	0.75
21	Lithium sulphate	0.50	0.25	1+	2−	1	0.75
22	Sodium sulphate	0.50	0.25	1+	2−	1	0.75
23	Ammonium sulphate	0.49	0.25	1+	2−	1	0.75
24	Magnesium sulphate	0.25	0.25	2+	2−	1	1.0
25	Potassium tartrate	0.50	0.25	1+	2−	1	0.75
26[d]	Sodium tartrate	0.50	0.25	1+	2−	1	0.75
27[d]	Potassium citrate	0.33	0.11	1+	3−	1	0.67
28[d]	Lithium citrate	0.33	0.11	1+	3−	1	0.67
29	Sodium citrate	0.33	0.11	1+	3−	1	0.67

[a] Used for identification of salts in figures and tables.

[b] Displacing ionic strength calculated by $I_D = m_{anion} z^2_{anion}$.

[c] Ionic strength in the eluting buffer calculated by $I = 0.5\sum(m_i z_i^2)$. The buffer concentration and protolytic equilibria are taken into account for this calculation only.

[d] Subset of salts selected for AEC at pH 9.6.

TABLE VI

SELECTION OF SALTS FOR CEC OF PEPTIDES AT pH 2.2

No.[a]	Salt	Molality		Valency		Ionic strength	
		m_{cation}	m_{anion}	z_{cation}	z_{anion}	I_D[b]	I[c]
1	Potassium chloride	1.0	1.0	1+	1−	1	1.0
2	Potassium phosphate	1.0	1.0	1+	1−	1	1.0
3	Lithium chloride	1.0	1.0	1+	1−	1	1.0
4	Lithium perchlorate	1.0	1.0	1+	1−	1	1.0
5	Lithium sulphate[d]	1.0	0.24, 0.38	1+	1−, 2−	1	1.4
6	Sodium chloride	1.0	1.0	1+	1−	1	1.0
7	Sodium perchlorate	1.0	1.0	1+	1−	1	1.0
8	Sodium phosphate	1.0	1.0	1+	1−	1	1.0
9	Sodium sulphate[d]	1.0	0.24, 0.38	1+	1−, 2−	1	1.4
10	Ammonium chloride	1.0	1.0	1+	1−	1	1.0
11	Ammonium phosphate	1.0	1.0	1+	1−	1	1.0
12	Ammonium sulphate[d]	1.0	0.24, 0.38	1+	1−, 2−	1	1.4
13	Calcium chloride	0.25	0.50	2+	1−	1	0.76
14	Magnesium chloride	0.25	0.50	2+	1−	1	0.76
15	Magnesium sulphate[d]	0.25	0.12, 0.19	2+	1−, 2−	1	0.94

[a] Used for identification of salts in figures and tables.
[b] Displacing ionic strength calculated by $I_D = m_{cation} z^2_{cation}$.
[c] Ionic strength in the eluting buffer calculated by $I = 0.5\sum(m_i z_i^2)$. The buffer concentration and protolytic equilibria are taken into account for this calculation only.
[d] Molality and valency stated for HSO_4^- and SO_4^{2-}, respectively.

spectively. This corresponds to making the salt concentration for a divalent displacing ion one quarter of the concentration of a monovalent ion (see Tables V and VI). Preparation of the eluting buffers with constant I_D ensures that all gradients have the same rate of increase in displacing ionic strength per unit volume. This should be contrasted with the apparent gradient slope, defined earlier in this paper, as the elution strength depends not only on the ionic strength but also on the identity of the displacing ion. The elution strength of a salt will hereafter refer, except where explicitly stated, to eluents with constant displacing ionic strength.

The ion exchanger will initially be loaded with a certain amount of counter-ions from the pH-adjusting reagent, as the column is equilibrated with the starting buffer. Complications due to mixed ionic forms of the ion exchanger may occur if the pH of the eluents is adjusted with the same acid or base for all salts. The pH of the eluents should therefore be adjusted with the acid or base corresponding to the displacing ion when possible. A certain amount of the displacing ion, determined by the buffer concentration and pH, will inevitably be present in the starting buffer. This leads to differences in the elution strength of the starting buffer, as discussed later in this paper.

The measured retention volumes for anion-exchange chromatography of the eight proteins at pH 8.0 are shown in Table VII. Two peaks were present for the protein amyloglucosidase, leading to a total of nine variables. To give an indication of the elution strength, the average retention volume for each salt is also tabulated. No absolute definition of the elution strength is available in IEC, but a higher elution strength ought to result in a lower average retention volume. As pointed out by Coenegracht *et al.* [6], however, the average retention volume is not an absolute measure of the elution strength only, because specific effects also influence the average retention volume.

In Table VIII the retention volumes for CEC of the nine peptides are shown together with the average retention volumes.

A subset of ten salts, indicated in Table VII, was selected to make a comparison between the effect of

TABLE VII

RETENTION DATA FOR AEC OF PROTEINS AT pH 8.0

Salt[a]	Retention volume (ml)[b]									
	Myo	CA	IgG	Tra	APh	BSA	STI	Amy1	Amy2	Average[c]
1	1.55	2.88	3.94	5.00	12.90	12.87	16.91	14.93	23.32	10.48
2[d]	1.67	2.88	3.86	5.01	13.03	12.98	16.98	14.86	23.22	10.50
3	1.57	3.16	3.70	4.83	12.00	11.91	16.14	13.96	22.47	9.97
4	1.74	2.94	3.25	4.20	8.31	8.35	10.19	10.02	14.70	7.08
5	1.80	2.97	3.37	4.48	9.44	9.37	12.23	11.06	17.49	8.02
6	1.66	3.30	3.90	5.17	8.75	8.73	8.98	8.76	10.95	6.69
7[d]	1.60	3.28	3.95	4.99	8.03	7.95	8.43	8.22	10.10	6.28
8	1.65	3.26	3.92	5.08	8.45	8.45	8.80	8.62	10.72	6.55
9	1.52	3.12	3.74	4.96	8.09	8.00	8.51	8.24	10.45	6.29
10[d]	1.63	3.11	3.24	4.15	5.71	5.75	5.47	5.77	6.75	4.62
11	3.04	3.24	3.97	5.13	10.05	9.95	10.30	10.15	13.39	7.69
12[d]	3.06	3.18	3.88	4.95	8.86	8.96	9.66	9.57	12.11	7.14
13[d]	1.44	2.90	3.73	4.93	9.33	9.26	9.88	9.80	12.70	7.11
14	1.33	3.28	3.62	4.83	8.88	8.82	9.52	9.37	12.28	6.88
15	1.48	2.83	3.08	3.91	5.96	5.93	5.71	6.19	7.44	4.73
16	1.51	2.83	3.17	4.13	6.34	6.34	6.54	6.68	8.30	5.09
17[d]	1.68	3.15	3.96	5.28	12.23	12.23	15.19	13.93	20.93	9.84
18	1.63	3.12	3.93	5.27	13.03	12.97	15.15	14.09	20.93	10.01
19	2.38	2.93	3.66	5.05	11.46	11.40	14.27	13.16	19.66	9.33
20[d]	1.15	1.31	3.24	3.69	10.10	10.05	13.61	13.27	21.56	8.66
21	1.14	1.31	3.20	3.58	9.06	9.04	12.08	11.74	17.69	7.65
22	1.14	1.29	3.17	3.67	9.70	9.62	13.19	12.93	20.47	8.35
23	1.13	1.30	3.20	3.65	9.08	9.09	12.74	12.28	19.49	8.00
24	1.13	1.32	3.01	3.60	7.92	7.83	9.36	9.16	14.17	6.39
25	1.09	1.30	3.25	4.00	10.72	10.74	14.44	13.96	23.15	9.18
26[d]	1.09	1.33	3.29	3.89	10.36	10.39	13.96	13.61	22.19	8.90
27[d]	1.02	1.08	3.38	1.36	6.85	6.74	11.41	11.48	20.02	7.04
28[d]	1.04	1.07	3.30	1.61	6.80	6.53	11.52	11.34	19.21	6.94
29	1.04	1.09	3.22	1.45	6.57	6.96	11.81	11.91	20.82	7.21

[a] For identification of salts, see Table V.
[b] For protein designations, see Table I.
[c] Average retention volume for each salt.
[d] Salts used for comparison with pH effect (see also Table IX).

a change in buffer pH and that of different displacing salts. The retention volumes of the proteins eluted with these salts at pH 9.6 are shown in Table IX.

Analysis of retention data for AEC of proteins at pH 8.0

The retention volumes for the nine protein peaks eluted with 29 salts at pH 8.0 were subjected to principal component analysis. The results of the analysis are summarized in Table X. The main causes of retention variations can be seen from the plot of the scores on the two first principal components (Fig. 4).

The most obvious pattern in the score plot is a clustering of the salts according to the displacing anion. It also can be observed that the scores on the two first principal components are related to the elution strength of the displacing salts. The average retention volume of the salts increases, corresponding to a decrease in elution strength, on going from the lower left to the upper right part of the score plot. The direction of decreasing elution strength correlates more closely with the first than the second component. On the other hand, a separation of the salts with monovalent anions from the multivalent salts can be seen in the scores on the second

TABLE VIII

RETENTION DATA FOR CEC OF PEPTIDES AT pH 2.2

Salt[a]	Retention volume (ml)[b]									
	p1	p2	p3	p4	p5	p6	p7	p8	p9	Average[c]
1	2.66	5.35	3.41	6.06	4.40	12.15	13.47	2.58	6.06	6.24
2	3.33	5.44	3.59	5.96	4.45	10.47	13.17	3.06	6.25	6.19
3	2.92	6.48	3.40	7.85	5.00	14.57	18.45	2.53	7.07	7.59
4	2.78	6.27	3.35	7.16	4.76	13.09	14.78	2.73	6.18	6.79
5	3.15	6.51	3.83	7.84	5.04	10.36	16.18	2.77	7.23	6.99
6	2.70	5.33	3.30	6.17	4.37	11.56	13.52	2.34	5.93	6.14
7	2.68	5.37	3.29	6.01	4.35	11.35	12.33	2.57	5.54	5.94
8	3.30	5.65	3.75	6.52	4.59	10.67	14.27	2.97	6.50	6.47
9	2.83	5.32	3.53	6.13	5.23	8.30	11.67	2.63	5.93	5.73
10	2.74	5.82	3.33	6.65	4.75	13.28	15.57	2.38	6.48	6.78
11	3.28	5.55	3.79	6.83	4.60	10.35	13.66	3.20	6.33	6.40
12	3.01	5.92	3.78	6.85	4.82	9.75	14.19	2.75	6.62	6.41
13	1.21	2.02	1.31	2.08	1.68	4.93	6.20	1.17	2.92	2.61
14	1.26	2.32	1.40	3.15	2.12	6.03	7.51	1.13	3.01	3.10
15	1.26	2.92	1.55	3.28	1.93	5.28	8.20	1.23	3.41	3.23

[a] For identification of salts, see Table VI.
[b] For peptide identification, see Table II.
[c] Average retention volume for each salt.

component. An interpretation of this is given later.

As stated previously, the primary clustering in the score plot involves the displacing anions. The salts with a common anion are subdivided, however, according to the valency of the accompanying cation. This indicates that the use of a constant displacing ionic strength does not compensate sufficiently for the difference in elution strength between salts with varying valencies of the constituent ions. The theory proposed by Ståhlberg *et al.* [17] states that the retention in isocratic IEC is related to the ionic strength in the eluting buffer (I), *i.e.*, the ionic

TABLE IX

RETENTION DATA FOR AEC OF PROTEINS AT pH 9.6

Salt[a]	Retention volume (ml)[b]								
	Myo	CA	IgG	Tra	APh	BSA	STI	Amy1	Amy2
2	5.39	5.96	8.25	7.67	16.42	16.32	18.53	16.73	24.73
7	4.11	4.04	5.47	6.08	8.90	8.78	8.72	8.62	10.34
10	3.98	3.64	4.44	4.82	6.27	6.25	5.62	6.18	6.96
12	4.42	4.04	5.75	6.23	10.16	10.08	10.12	10.17	12.64
13	4.26	3.89	5.80	6.32	10.73	10.71	10.57	10.68	13.34
17	4.67	4.32	8.74	7.56	15.56	15.47	16.00	15.04	21.56
20	1.49	2.63	4.61	5.02	13.63	13.69	14.92	15.07	23.37
26	1.75	1.58	4.87	5.12	13.72	13.56	15.46	15.72	24.11
27	1.12	1.09	3.18	3.33	10.35	10.33	12.77	13.38	21.98
28	1.11	1.10	3.12	3.25	9.43	9.33	12.70	12.92	20.78

[a] For identification of salts, see Table V.
[b] For protein designations, see Table I.

TABLE X

SUMMARY OF PCA RESULTS FOR ALL DATA SETS

All data sets are centred around the mean before the analysis.

Data set	Significant PCs[a]	Explained variance (%)[b]		
		PC1	PC2	PC3
AEC, all salts, pH 8.0	2	86.7	98.8	
CEC, all salts, pH 2.2	3	91.7	96.9	99.0
AEC, selected salts, pH 8.0	2	83.8	99.1	
AEC, selected salts, pH 9.6	2	81.7	98.9	
AEC, selected salts, pH 8.0 and 9.6	3	78.4	97.2	98.8

[a] Number of significant principal components according to cross-validation.
[b] Cumulative percentage of the total variance explained after each component.

strength calculated by considering all constituent ions. The displacing ionic strength and the ionic strength in the eluting buffer are given in Table V. On comparing the ionic strengths in the eluting buffers, different values are observed for salts with multivalent cations compared with those with a monovalent cation. The eluting buffers could have been prepared by keeping the ionic strength in the eluting buffer constant, instead of the displacing ionic strength. The resulting elution strength of the eluting buffer would then be lower for the salts consisting of a multivalent cation and a monovalent anion. This would lead to higher average retention volumes, moving them closer to the other salts with the same anion.

The same argument can be made for the salts with multivalent anions, *i.e.*, sulphates, tartrates and citrates. For the divalent anions, the calculated ionic strength in the eluting buffer is less than unity for all salts except magnesium sulphate. A constant ionic strength in the eluting buffer would lead to higher elution strengths for these salts having

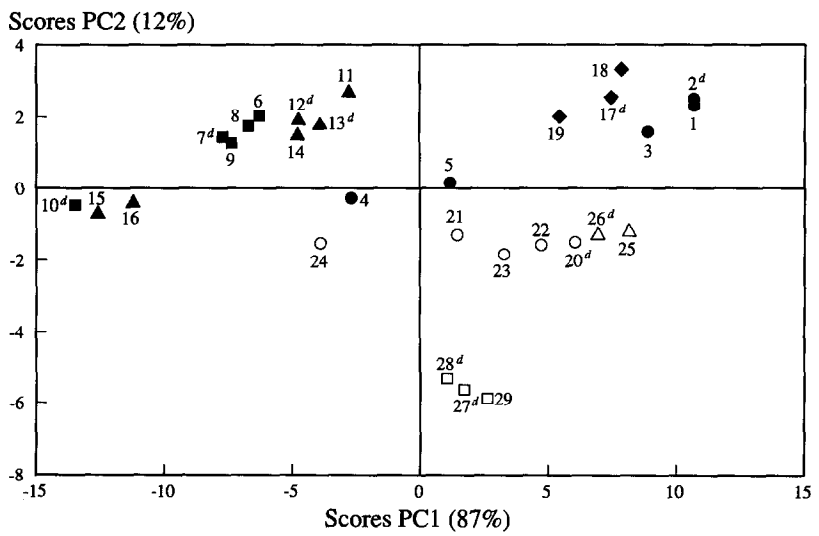

Fig. 4. AEC of proteins at pH 8.0. Score plot of the two first principal components (mean centred data). Unfilled symbols indicate multivalent anions: □ = citrates, △ = tartrates, ○ = sulphates. Filled symbols indicate monovalent anions: ■ = bromides; ▲ = chlorides; ● = acetates; ◆ = formates. For identification of the individual salts, see Table V. [d] Indicates salts selected for comparison with pH 9.6.

monovalent cations, moving them closer to magnesium sulphate. For the trivalent anion citrate, all salts have a lower ionic strength in the eluting buffer than the other salts. By preparing a stronger eluent, keeping the ionic strength in the eluting buffer constant, the citrates would move further to the lower left in the score plot. This agrees with the empirical eluotropic series [18], shown in Table XI, where citrate is listed as the strongest displacer in AEC.

The essence of this discussion is that the eluting buffers should be prepared with the same ionic strength in the eluting buffer for all salts. By instead keeping the displacing ionic strength constant, the resulting elution strength varies between salts with different valencies for the co-ion. This is reflected in the average retention volumes, listed in Table VII, and is also evident in the score plot in Fig. 4.

The second principal component shows a division of the salts mainly according to the valency of the displacing anion. This can be attributed to some extent to differences in the elution strength of the starting buffer. The ionic strength in the starting buffer is higher for salts with multivalent anions compared with salts with monovalent anions. This is an inherent consequence of the pH adjustment of the buffers, as it was done with the acid corresponding to the displacing anion. The approximate ionic strength in the starting buffers used in this part of the study is shown in Table XII for mono-, di- and trivalent anions. These differences in ionic strength exist also during the gradient, but become negligible owing to the increase in the salt concentration. A higher ionic strength at the start of the gradient leads to earlier elution of the moderately retained proteins. The elution strength in the starting buffer is not constant even for salts with the same valency of the anion. The starting eluent is thus stronger for bromides than for acetates. The magnitude of this influence is small, however, compared with the effect of the valency of the anion.

TABLE XI

IONS ARRANGED IN ORDER OF INCREASING ELUTION STRENGTH

Data adapted from ref. 18.

AEC	Acetate < formate < chloride < bromide < sulphate < citrate
CEC	Lithium < sodium < ammonium < potassium < magnesium < calcium

TABLE XII

IONIC STRENGTH IN THE STARTING ELUENT FOR AEC AT pH 8.0

10 millimolal Tris buffer (pK_a = 8.06).

pH adjusted with	Ionic strength
Monovalent acid	0.0053
Divalent acid	0.0080
Trivalent acid	0.0107

The first principal component is dominated by the peaks with high retention volumes, *i.e.*, Amy2, STI and Amy1. This is illustrated in the loading plot for the two first principal components in Fig. 5. The variations in the apparent gradient slope resulting from the different elution strengths of the salts influence the late-eluting peaks to a larger extent than peaks at the beginning of the chromatogram. The peaks with small retention, *i.e.*, CA, Myo, Tra and IgG, have very low loadings on the first component. These peaks are mainly influenced by the elution strength during the beginning of the gradient. Their relatively larger loadings on the second principal component support the interpretation of the second component as primarily a measure of the elution strength in the starting buffer.

The impact of the displacing salts on the retention behaviour of proteins in AEC can be summarized by two principal components, explaining about 99% of the retention variations. Two major contributions to the influence of the displacing salt can be identified, the apparent gradient slope and the elution strength of the starting buffer. These two effects are not independent of each other, as a strong displacer gives both a high apparent gradient slope and a high elution strength in the starting buffer. This is also evident in the score plot as the direction of increasing elution strength of the salts is not orthogonal to the second principal component, associated with the elution strength in the starting buffer. Instead, this direction is tilted downward towards the region of multivalent anions, where the elution strength in the starting buffer is high.

If retention data from duplicate analyses of some salts are included in the PCA, only negligible devia-

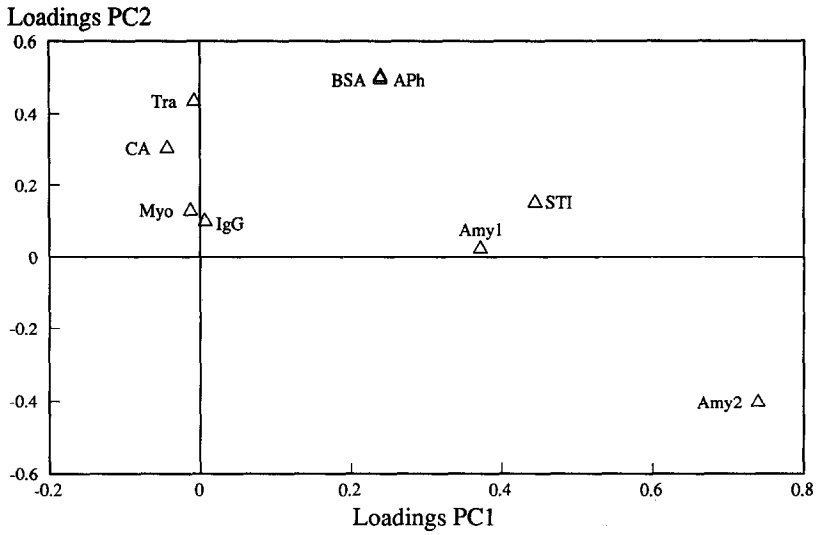

Fig. 5. AEC of proteins at pH 8.0. Loading plot of the two first principal components. For protein designations, see Table I.

tions from the pattern in Fig. 4 are present (data not shown).

Analysis of retention data for CEC of peptides at pH 2.2

The results from the PCA of the cation-exchange data are summarized in Table X. The primary clustering in the score plot of the two first components (Fig. 6) corresponds to the valency of the displacing cation. The salts with divalent cations, *i.e.*, calcium and magnesium, are separated from the other salts that have lower elution strengths. The direction of decreasing elution strength is more or less parallel to the first principal component in this case. By lin-

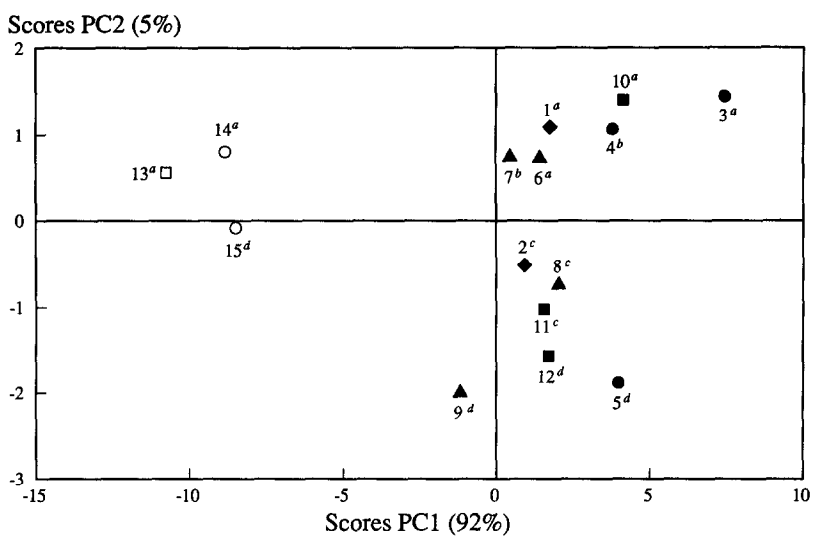

Fig. 6. CEC of peptides at pH 2.2. Score plot of the two first principal components (mean centred data). Unfilled symbols indicate multivalent cations: □ = calcium; ○ = magnesium. Filled symbols indicate monovalent cations: ■ = ammonium; ▲ = sodium; ● = lithium; ◆ = potassium. The anions are indicated by superscript letters: [a] chlorides; [b] perchlorates; [c] phosphates; [d] sulphates. For identification of the individual salts, see Table VI.

ear regression of the average retention volumes as a function of the scores on PC1 a good correlation ($r = 0.994$) can be demonstrated.

In contrast to the AEC experiments, the pH adjustment of the starting buffer was done with a monovalent base, lithium hydroxide, for the salts with divalent cations. This was necessary because of the limited solubility of magnesium hydroxide and calcium hydroxide. As a consequence, only small differences in the elution strength of the starting buffer are present. This explains the absence of a segregation induced by the pH adjustment procedure. The secondary grouping of the salts with monovalent cations is instead related to the accompanying anion, with the sulphates and phosphates separated from the others. Two possible interpretations of this observation will be discussed, although no definitive conclusion can be drawn regarding the actual source of the secondary grouping. It also should be pointed out that the first component explains the main part of the total variations in retention, so the contribution of the second component is relatively small.

In IEC, the pH of the eluate fluctuates during the gradient [19], especially when low buffer concentrations are used as in this study. The pH variations will in this instance affect not only the charge of the solutes but also the charge of the ion exchanger, as a Mono S column exhibits a certain buffer capacity at pH 2.2, in spite of being a strong cation exchanger [20]. The appearance of these pH variations will be different when the gradient is formed with a salt that has an appreciable buffer capacity at the given pH. The secondary grouping may reflect the differences in buffer capacity of the anions, as sulphates and phosphates have the highest buffer capacity at pH 2.2. However, it is not possible to elucidate the precise influence of the pH fluctuations on the individual peptides.

Another possibility for interpretation of the secondary grouping arises from the small hydrophobic interactions that may occur in spite of the presence of 30% of acetonitrile in the mobile phase. The amount of acetonitrile added was chosen to suppress the hydrophobic interactions totally, but at high salt concentrations they still might not be negligible. The chaotropic effect of an ion is the ability to make a solvent less polar. A chaotropic ion has *salting-in* properties, suppressing the hydrophobic interactions even further. An ion with the opposite, *salting-out*, properties is termed kosmotropic and promotes hydrophobic interactions. Kosmotropic ions are known to stabilize protein structure [21], as opposed to the destabilizing properties of chaotropic ions. The chaotropic character of ions has been related to the lyotropic series of Hofmeister that expresses the ion-specific influence on protein solubility [22]. The ions used in this work for CEC are listed according to increasing chaotropic character in Table XIII. By using salts with a kosmotropic anion, *e.g.*, sulphate or phosphate, more hydrophobic interactions are introduced. The grouping of the salts according to the anions may be interpreted in these terms, as the difference in chaotropic character is larger for anions than for cations [4]. The influence of the chaotropic character would be most pronounced for the late-eluting peaks, where the salt concentration is high. Further, the largest effects ought to be seen for hydrophobic peptides as they are more prone to hydrophobic interactions.

The loading plot of the two first principal components is shown in Fig. 7. The first component is dominated by the two late-eluting peptides, p7 and p6. The influence of the individual peaks on PC1 is roughly related to their retention volumes. This is indicative of the correspondence of the first component to the apparent gradient slope. The second component is mainly related to one peptide, p6, with only small contributions from the other peptides. This peptide is indeed eluted at high salt concentrations, but it is not very hydrophobic (see Table II). As a consequence, the loading plot contradicts the interpretation of the secondary grouping as being due to hydrophobic interactions. On the other hand, the loading plot does not make it possible to conclude that the secondary grouping arises from the buffer capacity either. This is prevented by the absence of information regarding the actual effect of pH variations during the gradient.

TABLE XIII

IONS ARRANGED IN ORDER OF INCREASING CHAOTROPIC CHARACTER

Data adapted from refs. 18 and 23.

Anions	Phosphate < sulphate ≤ chloride < perchlorate
Cations	Calcium < magnesium ≤ lithium < sodium < potassium < ammonium

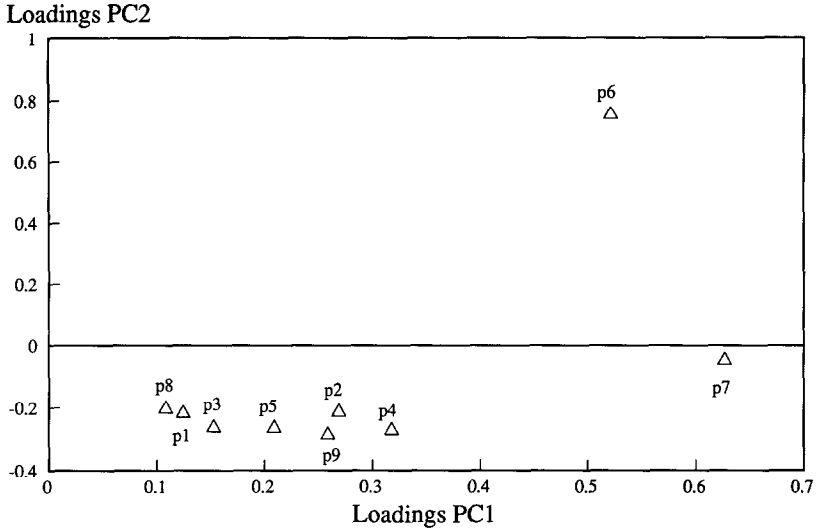

Fig. 7. CEC of peptides at pH 2.2. Loading plot of the two first principal components. For peptide designations, see Table II.

The two first principal components explain about 97% of all variations in the retention volumes. In the absence of substantial differences in the elution strength of the starting buffer, the retention variations depend on the apparent gradient slope to a greater extent compared with the AEC data. The observed secondary grouping according to the anions reflects only a minor contribution to the overall variations, and the cause of it cannot be elucidated.

Practical consequences

The influence of different displacing salts can be divided into non-specific and specific effects. The non-specific effects can be regarded as differences in the apparent gradient slope. On the other hand, the specific effects alter the retention differently for individual peaks. The influence of the elution strength of the starting buffer cannot accurately be regarded as a specific effect, although it affects the retention for the early-eluting peaks only. It can be distinguished from specific effects as all early-eluting peaks will be affected in the same manner. Further, all salts giving the same elution strength at the start of the gradient will have the same influence.

The main effects on retention seen in this work can be explained by differences in the gradient, *i.e.*, the combined effect of the elution strength in the starting buffer and the apparent gradient slope. This disagrees with some of the results of other workers [2,3], where the large selectivity differences demonstrated are attributed to specific, salt-mediated effects. It should be pointed out that the data presented in this paper also include large effects on the selectivity. To illustrate this, the retention ratio between the peaks Amy2 and BSA in AEC at pH 8.0 was calculated for all salts. The values ranged from 1.2 (for calcium bromide) to 3.0 (sodium citrate). The range became narrower (1.3–1.9) when the calculations were restricted to salts having the same ionic strength in the eluting buffer. The differences in selectivity shown by this example are nevertheless to a large extent explained by the elution strength in the starting buffer and the apparent gradient slope.

It must be emphasized that the outcome of the principal component analysis reflects the design of the data set, *i.e.*, the experimental conditions chosen for the compilation of the data set. In this paper, we have presented results from experiments performed by choosing the most intuitive experimental conditions, *e.g.*, regarding the concentration of the eluting buffer and pH adjustment procedure. This makes it possible to relate our results to those in previous work regarding displacing salts. As a consequence, the relatively small specific effects on the

retention that may be present are buried by the large impact on retention due to differences in the elution strength in the starting buffer and the apparent gradient slope. To be able to evaluate the possible specific effects of the displacing salts, another strategy is necessary. By maintaining constant apparent gradient slope and elution strength in the starting buffer for each salt, the influence of the non-specific effects of the salts will be reduced. The score plots, resulting from the principal component analysis of this modified data set, will thus reveal the true specific effects of the displacing salts on the retention. The adjustment of the concentrations can be accomplished by trial and error, or preferably by using the theory of gradient elution in IEC [24,25] allowing the prediction of retention at any gradient from two or more initial gradients.

Comparison of the influence of pH and displacing salts

As pointed out previously, the pH of the buffers is the most obvious property to alter when developing a separation method. This led to an attempt to compare the effect of pH changes, although within a limited range, with the influence of different displacing salts.

Ten salts were selected from diverse parts of the score plot for AEC at pH 8.0 (see Fig. 4). The results from the principal component analysis of this subset of salts are summarized in Table X. The pattern of the salts in the score plot of the two first components is easily recognizable (data not shown), looking very much the same in this subset as in the complete set of salts.

The salts included in the subset were used for elution of the proteins using a piperazine buffer at pH 9.6. The retention data presented in Table IX were subjected to PCA (see Table X). The score plot (data not shown), reveals the same general pattern as at pH 8.0. The close resemblance of the two score plots indicates that the characterization of the retention behaviour made at pH 8.0 may also be valid at other pH values.

The retention data for the selected subset of salts at the two pH values can be joined together and analysed by a joint principal component analysis (see Table X). The score plot of the two first components is shown in Fig. 8. An increase in pH from 8.0 to 9.6 generally results in an increase in the scores on both the first and second component, leading to a movement upwards and slightly to the right in the score plot. It should be pointed out that the two principal components do not reflect the same combination of the original variables as in the PCA of

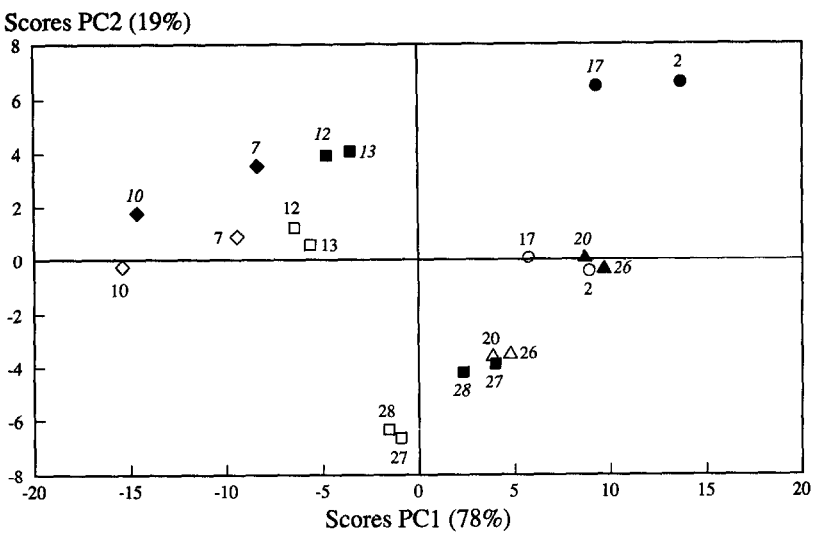

Fig. 8. AEC of proteins at pH 8.0 and 9.6. Score plot of the two first principal components using the selected subset of salts (mean centred data). Unfilled symbols indicate data at pH 8.0: □ = citrate and chloride; △ = sulphate and tartrate; ○ = formate and acetate; ◇ = bromide. Filled symbols and salt designations in italics indicate data at pH 9.6. For identification of individual salts, see Table V.

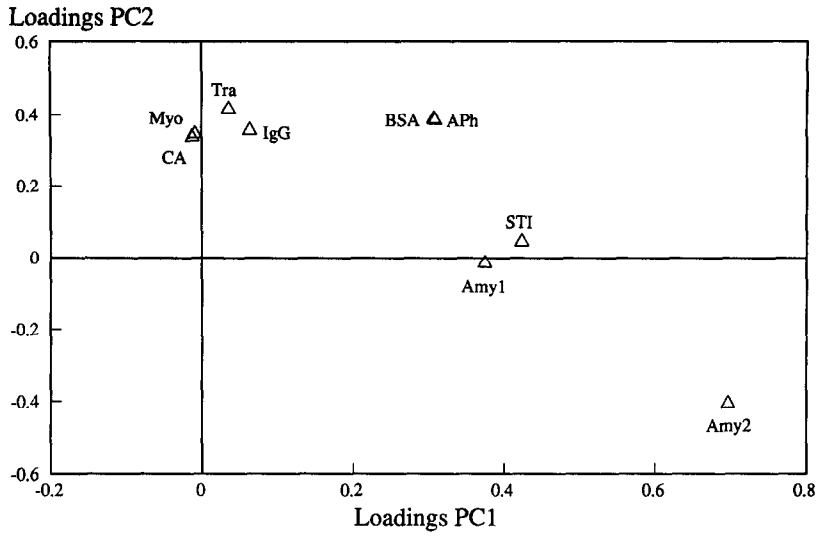

Fig. 9. AEC of proteins at pH 8.0 and 9.6. Loading plot of the two first principal components. For protein designations, see Table I.

the pH 8.0 data only. This implies that the direction of movement when the pH is changed cannot be interpreted in terms of apparent gradient slope or elution strength of the starting buffer. If the joint loading plot (Fig. 9) is compared with the pH 8.0 loading plot (Fig. 5), the most obvious deviation is the increase of the loadings for Myo, CA and IgG on the second component. It is difficult to establish the specificity of the influence of pH changes, but the loading plot supports the self-evident notion that the proteins with isoelectric points closest to 8.0 are relatively more affected by an increase in pH.

It is interesting that the influence of the displacing salts is of the same magnitude as the effect of changing the pH in this limited range, as shown by the score plot. This corroborates the comment of Kopaciewicz and Regnier [2] that the displacing salt is equally important as the pH for controlling retention and selectivity. However, if a broader pH range is considered, greater effects of pH changes will be observed. The retention will be drastically reduced for proteins that are positively charged at the lower pH.

CONCLUSIONS

Principal component analysis has proved to be useful for the characterization of retention behaviour. In applying this method to the ion-exchange chromatography of peptides and proteins, several interesting observations can be made.

A change of the displacing salt has major effects on the retention. If the eluting buffers are prepared with constant ionic strength, which is appropriate, a change of displacing salt will influence the retention in two ways. First the apparent gradient slope, *i.e.*, the increase in elution strength per unit volume, will be affected. Second, the elution strength of the starting buffer will change. With a constant concentration of the buffer substance, the ionic strength in the starting buffer will inevitably vary with the valency of the displacing ion, provided that the pH adjustment is done appropriately. The elution strength in the starting buffer will therefore be higher for multivalent displacing ions at the same buffer concentration. This leads to earlier elution of the moderately retained peaks with multivalent displacing ions, despite a constant ionic strength in the eluting buffer.

An important conclusion is that the main effect of a change of the displacing salt is non-specific. The chromatograms may look very different with different salts, but this is often solely caused by a change in the apparent gradient slope and elution strength of the starting buffer. The observed alterations in retention volumes when changing the displacing salt could often be achieved by modifica-

tions of the gradient with the original salt.

In this work a fairly large number of proteins and peptides were tested. However, all possible variations can never be covered. This study aimed to be more general than previous work, but it must be pointed out that very specific effects are nevertheless possible with individual proteins and peptides. Drastic effects on the selectivity by a change of displacing salt could be caused by alterations in the higher structure of a protein. For many applications this would hardly be a desirable way to adjust the selectivity. Another possibility for specific effects is the promotion or suppression of hydrophobic interactions caused by the chaotropic character of the displacing salt.

Retention control is not the only relevant property of the displacing salt. The displacing salt also affects the solubility and stability of proteins and peptides. In addition, practical considerations, such as whether the salt is corrosive or can be mixed with organic solvents, have to be included. These practical considerations might be more important than selectivity control when selecting the displacing salt.

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