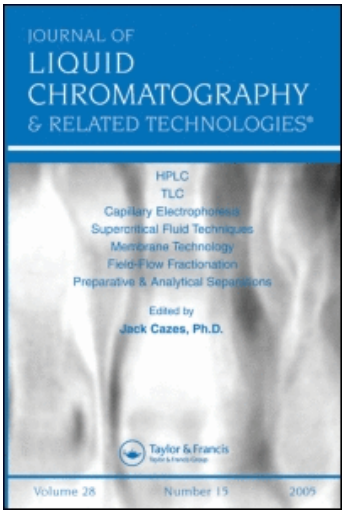

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Optical Resolution by Liquid Chromatography on Immobilized Bovine Serum Albumin

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OPTICAL RESOLUTION BY LIQUID CHROMATOGRAPHY ON IMMOBILIZED BOVINE SERUM ALBUMIN

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ABSTRACT

Direct optical resolution by liquid chromatography based on the enantioselective properties of a protein, particularly bovine serum albumin (BSA), has been shown to be a very versatile method with many useful analytical applications. Although the mechanism of chiral recognition by the protein is largely unknown, some empirically found correlations between retention behaviour and mobile phase composition give a general idea of the main types of solute-protein interactions involved. A summary of results from optical resolution of different classes of racemic compounds is given, together with examples of large substituent effects on retention values.

INTRODUCTION

Liquid chromatographic methods based on the use of chiral stationary phases (CSP's) have attracted a great deal of interest from theoretical as well as practical points of view as means to achieve direct optical resolution. The various principles used and the progress made in the field can be found in a number of recently published review articles (1-7) and will not be dealt with here. However, the increased complexity of

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chiral recognition phenomena which results from going from low molecular weight chiral selectors to CSP's based on biopolymers or synthetic chiral polymers of high molecular weight should be considered. While in the first case a chiral recognition rationale may be postulated and found useful in predicting the elution order of the enantiomers, as found in ligand exchange chromatography (8), crown ether complexation (9) or with the CSP's developed by Pirkle (10), this is often not possible for macromolecular CSP's with a complex tertiary structure. The important role of the latter is, however, well-known from e.g. optical resolutions on acetylated cellulose, where conservation of the microcrystallinity of the material is crucial (11). Similarly, the isotactic poly-triphenylmethyl methacrylate obtained via chiral anion polymerization (12) forms a helical coil structure, the chirality of which is responsible for the enantioselectivity observed. Proteins are even more complex since the polypeptide chains are built up from amino acids with varying α-substituents yielding tertiary structures involving complicated chain folding, disulfide bridging, as well as intra- and interchain non-covalent bonding interactions. Despite the difficulties in rationalizing the ligand binding properties and enantioselective action of a protein, biomolecules of this kind are nevertheless of great interest as CSP's in HPLC-columns for optical resolution purposes. It is the aim of this paper to describe in more detail how an albumin, viz. bovine serum albumin (BSA), functions as a CSP in analytical HPLC with respect to optical resolution of a variety of structurally different racemic compounds.

BACKGROUND - STRUCTURE OF BSA AND STUDIES OF ITS BINDING PROPERTIES

BSA is a globular protein of mol.wt. 66210, consisting of 581 amino acids in a single chain, 17 intrachain disulfide bridges connect the 34 half-cystines with the formation of nine double loops (13). It is a relatively acidic protein (isoelectric point 4.7),

highly soluble in water, but like most albumins precipitates from solution at high salt concentration (14). Its net charge at pH 7.0 is -18. The over-all hydrophobic character of BSA is evident from various types of investigations, such as studies of the increased solubility of organic solvents, e.g. benzene, in aqueous solutions of albumin as compared to water itself (15). Numerous examples of binding of organic compounds to albumins are known (16) and it has been suggested that any organic compound with a hydrophobic contribution of at least five to six - CH_2 units binds to albumins (17). Quite clearly, hydrophobic interaction represents an important contribution to the total affinity of organic ligands for BSA.

A general phenomenon found in early studies on BSA is its ability to bind anions, inorganic as well as organic, but not cations unless there is a large hydrophobic contribution to the latter. In fact, di- or trivalent inorganic cations are known to cause destabilization of the albumin structure (18). The effect is supposed to be due to weakly repulsive interaction between cations and the hydrophobic surface.

Although hydrophobic interaction is often important, there are of course other contributions to consider, mainly electrostatic (Coulombic) interactions, hydrogen bonding and charge-transfer processes.

Probably the first observation of the enantioselective properties of an albumin was made by McMenamy and Oncley in 1958 who deduced from radioisotope labelling experiments and using an equilibrium dialysis technique (19) that the affinity for L-tryptophan exceeded that of the D-enantiomer by a factor of ca. 100. This observation was later followed by others, all based on studies of equilibria in solution by various techniques and this topic has recently been reviewed (20). The first direct confirmation of the results from 1958 concerning enantioselective binding of D, L-tryptophan was made in 1973 when Stewart and Doherty demonstrated that the D-enantiomer was eluted under conditions where the L-form was highly retained during affinity

chromatography on BSA immobilized to Sepharose (21). Some years later Lagercrantz et al. first studied the separation of racemic ^{14}C-warfarin into enantiomers by this technique (22) and then also the behaviour of D, L-tryptophan and (±)-warfarin on columns with immobilized serum albumins from different species (23). Systematic investigations of the chromatographic behaviour of various racemic compounds under different mobile phase conditions using continuous UV and electrochemical detection was initiated in 1982 (24) followed by the introduction of BSA-silica for HPLC columns (Resolvosil® - BSA, now available from Macherey-Nagel GmbH, Düren, FRG) a year later (25).

RETENTION BEHAVIOUR AND ENANTIOSEPARATION OF VARIOUS TYPES OF RACEMIC SOLUTES

Charged solutes

Quite generally the retention of charged, as opposed to non-charged, racemic solutes is more influenced by changes in pH and ionic strength of the mobile phase. This is not surprising if one considers the important role of electrostatic interactions in this case, causing the protein to act partly as a weak ion-exchanger. Consequently, the negative net charge of BSA will decrease with decreasing pH down to its isoelectric point, an effect which drastically increases the retention of negatively charged solutes such as carboxylates. Conversely, the effect on positively charged solutes like protonated amines and even zwitterionic amino acids appears to be the opposite.

A. Carboxylic acids. Fairly detailed studies on the optical resolution of various N-acylamino acids (25) under varying mobile phase conditions have been made. Decreasing retention of both enantiomers with increasing pH will often be accompanied by an increase in α, which may reach very high values (for p-nitrobenzoyl-alanine an α of ~17 has been obtained). Other

N-derivatives of amino acids found to be resolvable include benzenesulfonyl- (26), phthalimido- (26), 5-dimethylamino-1-naphthalenesulfonyl-(DANSYL-) (27), 2,4-dinitrophenyl-(DNP-) (27), and 2,4,6-trinitrophenyl-(TNP-) (27) derivatives. In the series investigated, the enantiomeric elution order is unchanged irrespective of the N-substituent (benzoyl, DANSYL, DNP). Fig. 1 shows the resolution of DNP-D,L-glutamic acid into its enantiomers. One carboxylic acid containing a sulfoxide function as the chiral centre, viz. 2-carboxyphenyl methyl sulfoxide (2-methylsulfinylbenzoic acid), has also been studied. It was found that the S(-)-enantiomer eluted prior to the R-(+)-form at all mobile phase compositions used (28). As for other acids studied, retention of both enantiomers was found to increase drastically with decreasing ionic strength. These and other effects of the mobile phase will be treated in more detail in a later section.

B. Amines. Only a few racemic amines have been investigated thus far. As might be expected however, retention, and also α, was found to increase with increasing pH. As the useful pH-limit on the alkaline side is probably set by the silica support (increased solubility) and not by the protein (risk for denaturation) the full use of this CSP is hampered by the limited use of silica-based stationary phases with alkaline mobile phases. Prilocain, (±)-2-(propylamino)-o-propiono-toluidide, a local anesthetic (Astra Pharm.Co.), for example, showed an α-value of 1.6 in 50 mM phosphate buffer, pH 8.9, with no added 1-propanol.

C. Aromatic amino acids. In addition to D,L-tryptophan, 5-hydroxy-D,L-tryptophan, D,L-kynurenine and 3-hydroxy-D,L-kynurenine were also found to be optically well resolved (24). The L-forms of these compounds are all well-known members of the tryptophan metabolism and of significant physiological importance. Like the situation for amines,

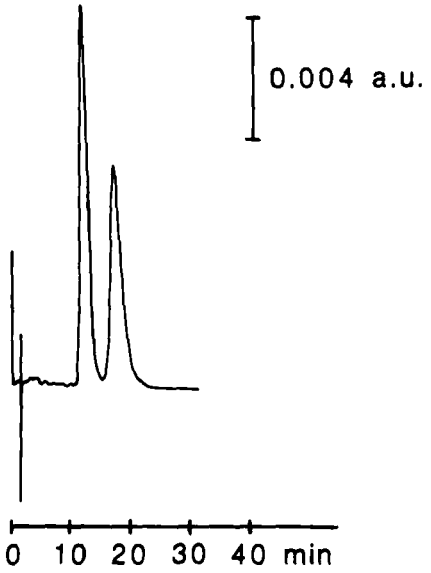


FIGURE 1. Resolution of DNP-D, L-Glutamic Acid. Mobile phase: 50 mM phosphate buffer with 2% 1-propanol, pH 7.3; flow rate: 2 ml/min; detection: UV 340 nm.

α-values tend to increase with increasing pH of the mobile phase and the effect on k' is largest for the last eluted enantiomer. No extreme pH-values are needed for good resolution, however. Thus, at pH 6.9 (0.05 M phosphate buffer) D, L-kynurenine resolves with an α-value > 9 (29).

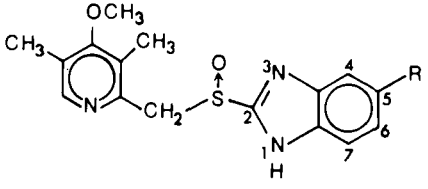
Non-charged solutes

A variety of compounds of this category, i.e. not taking part in protolytic equilibria, have been found to interact with the BSA CSP in an enantioselective mode. These include a number of esters of N-substituted amino acids, which generally show less retention than the free acids under identical chromatographic conditions. As will be shown later this represents an ideal situation for studies of stereospecific enzyme action on racemic ester substrates.

A. Sulfoxides, sulfoximine derivatives and related sulfur compounds. There are good reasons to believe that many alkyl aryl sulfoxides are optically well resolved, although thus far only a very limited number of sulfoxides with rather special substituents has been investigated. A series of substituted 2-pyridinylmethyl 2-benzimidazolyl sulfoxides (I) of pharmacological importance has been studied with respect to substituent and mobile phase effects upon retention and resolution (30). It was found that in I the size of a substituent in the 5-position had a very marked effect, as shown in Table 1.

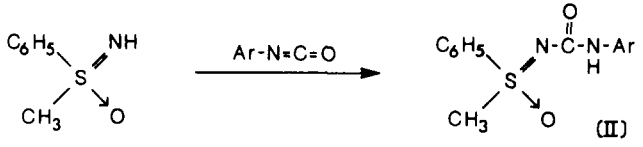
Substituted sulfoximines of structure II below are readily obtained by the reaction of methyl phenyl sulfoximine with an aryl isocyanate.

Just as in the sulfoxides the oxygen atom at the chiral pyrimidal sulfur centre is likely to interact with a protonated amine function in BSA. In this situation the remaining substituents are left in a chiral environment by the protein, yielding different contributions to the total binding energy for the two enantiomers. The capacity of immobilized BSA to



R= Cl (Ia)
Br (Ib)
$CH(CH_3)_2$ (Ic)
$C(CH_3)_3$ (Id)

SCHEME 1. Structures of Compounds I a-d



SCHEME 2. Synthesis and Structures of Compounds II

TABLE 1

k′- and α-Values of Sulfoxides I a-d under Identical Chromatographic Conditions

Compound	k_1'	k_2'	α
I a	11.7	26.9	2.3
I b	20.9	57.4	2.7
I c	7.3	47.4	6.5
I d	10.4	78.5	7.5

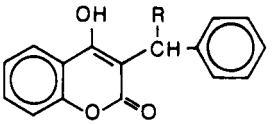
Mobile phase: 50 mM phosphate buffer, 2% 1-propanol, pH 6.0

resolve compounds of structure II was earlier demonstrated for Ar = C_6H_5 (28).

B. Coumarin derivatives. Two common racemic drugs, phenprocoumon (III a) and warfarin (III b), respectively, are known from binding studies in solution to interact enantioselectively with serum albumin (31). This has been definitely confirmed by chiral chromatography on immobilized BSA. Resolution of compound III b is shown in Fig. 2.

C. Benzodiazepine derivatives. A number of compounds of the benzodiazepine type (IV) are of pharmacological importance as sedative drugs. In order to study the effect of the substituent R attached directly to the asymmetric carbon atom, the series of compounds IV a-g was investigated. As shown in Table 2 there is a clear correlation between the hydrophobicity of R and the retention values.

D. Other compounds. Although only a very limited number of compounds have been investigated, some of the results indicate a broader usefulness of the BSA CSP, and may be



R= CH_2CH_3 (IIIa)
CH_2COCH_3 (IIIb)

SCHEME 3. Structures of Compounds III a-b

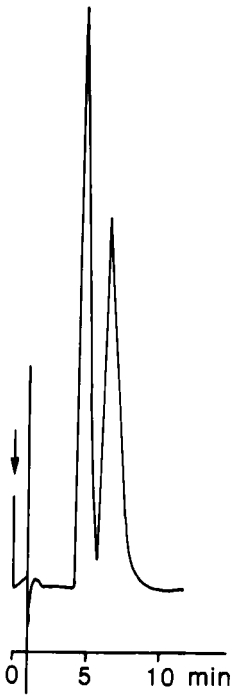
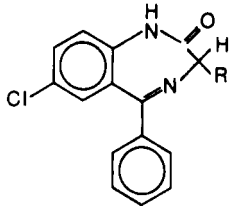


FIGURE 2. Resolution of Warfarin. Mobile phase: 50 mM phosphate buffer with 3% 1-propanol, pH 6.8; flow rate: 2 ml/min; detection: UV 225 nm.



R= OH (IVa)
CH_3 (IVb)
$CH_2CH_2CH_2CH_3$ (IVc)
$CH_2C_6H_5$ (IVd)
$CH_2CH_2CH_2OH$ (IVe)
$OCOCH_2CH_3$ (IVf)
$OCH_2CH_2CH(CH_3)_2$ (IVg)

SCHEME 4. Structures of Compounds IV a-g

TABLE 2

k′- and α-Values of Benzodiazepine Derivatives IV a-g

Compound	Mobile phase composition Molarity, mM	pH	1-propanol, %	k'_1	k'_2	α
IVa	10	6.6	2	2.1	8.6	4.10
IVb	50	7.8	6	5.1	5.1	1.00
	20	7.5	1	7.25	14.7	2.03
IVc	50	7.8	6	11.1	17.4	1.57
IVd	50	7.8	6	41.0	110.0	2.68
IVe	50	7.8	6	1.5	1.65	~1.09
	20	7.5	1	3.0	5.0	1.67
IVf	50	7.8	6	3.2	4.7	1.47
	20	7.5	1	6.5	10.75	1.65
IVg	50	7.8	6	14.0	28.2	2.01

of interest. It was found that the ethyl ester of N-acetyl-D, L-tryptophan could be completely separated into its enantiomers, both of which elute prior to the antipodes of the free acid (32). Since analogues results were obtained for some DNP-amino acid methyl esters (27), there is reason to believe that quite a number of similar racemic amino acid derivatives are resolvable.

Furthermore, benzoin (α-hydroxybenzyl phenyl ketone) should be mentioned which despite its structural simplicity is resolved with an α-value ~ 1.5.

SOLUTE STRUCTURE AND ENANTIOSELECTION - PREREQUISITES FOR CHIRAL RECOGNITION

It has been established that hydrophobic and electrostatic (Coulombic) interactions are the two most important factors contributing to retention, although hydrogen bonding and charge-transfer interaction may also be of significance. Thus, it was shown by Porath in 1978 that chromatography of tryptamine on DNP-S-Sephadex gives a moving coloured zone due to charge-transfer interaction (33). A similar situation might be present when compounds with electron accepting groups are separated on BSA-silica. This is due to the presence of two tryptophanyl residues in the protein and may account for the observation that retention tends to increase with the degree of nitro substitution in N-nitrobenzoyl- and N-nitrophenyl-amino acids.

Accordingly, a prerequisite for optical resolution on BSA-columns, in view of the types of interactions involved, should be the presence of aromatic as well as relatively polar groups in the racemic compound. Steric effects also appear to be highly important, similarly to what is usually found in enzyme-substrate interactions, and account for the largely unpredictable results from substituent variation (cf. Table 2.).

MOBILE PHASE EFFECTS

Many hydrophobic compounds, like N-naphthoylamino acids, are highly retained on BSA-columns, and their elution will require a few percent of a mobile phase modifier such as 1-propanol, in order to reduce the hydrophobic interaction and to obtain practically useful k′-values. As previously demonstrated, this is a very effective means of regulating retention (25, 30, 34) and 1-propanol concentrations $< 8\%$ are usually sufficient.

The effects from the pH of the mobile phase have already been mentioned, being very large with charged solutes. A useful pH-range, within which BSA-columns can be operated via phosphate and borate buffer systems, is 4-9. The characteristic feature of BSA not to bind organic cations, unless they are very hydrophobic, is reflected in the low retention values found for amines and free amino acids, including tryptophan, on BSA-columns in acidic buffer media.

A third mobile phase parameter to consider is the ionic strength. k′-values of carboxylic acids can be increased dramatically by a decrease in buffer concentration from 50 to 10 mM. This is most likely caused by effects on electrostatic interactions. It was recently demonstrated, however, that at buffer concentrations > 200 mM a reversal of the effect on k′-values of a series of N-benzoylamino acids occurred (34), indicating a greater influence on hydrophobic interaction in this buffer concentration range.

These mobile phase parameters can all be used to optimize an optical resolution. As found in Table 2 for compound II b, a mobile phase change, essentially involving a decrease in 1-propanol content and buffer strength, will increase the α-value from unity to 2.03 due to its large effect on k'_2.

SOME ANALYTICAL APPLICATIONS

As mentioned previously, the binding properties of BSA are highly dependent upon the aqueous buffer system used, partly due to a change in the protein itself (net charge, conformation). As a consequence of this, it is often possible to optimize a separation by a proper selection of mobile phase parameters, which may be particularly important in studies of stereoselective chemical conversions where both reactants and products are present in the analyzed sample. Further, using an aqueous mobile phase allows a direct injection of aqueous samples onto the column, which facilitates studies of enzymatic reactions and related biochemical processes (32) as the need for extractive isolation of reaction products is often eliminated. Recently, a study of the application of this technique to monitor stereoselective microbial conversions of certain racemic substrates was initiated and shown to be very useful (35). An example is illustrated by Fig. 3. The L-form of N-benzoyl-D, L-alanine is degraded faster than its optical antipode by the bacterium Nocardia restrictus. A microbial enzyme hydrolyzing the amide bond is likely to be responsible for this effect because benzoic acid appears as an intermediate in the process. The effect of micro-organisms on carboxylic ester substrates may be studied analogously, with the additional advantage that the carboxylic acid produced will be more retained and be readily identified. This is illustrated by Fig. 4, which shows the remarkable enantioselective action of the bacterium Arthrobacter oxydans upon acetyl-D, L-tryptophan ethyl ester (36).

Due to the large number of amino acid derivatives that has been shown to be resolvable, a further area of general significance would be the analysis of amino acid racemization, an important problem in peptide and protein chemistry.

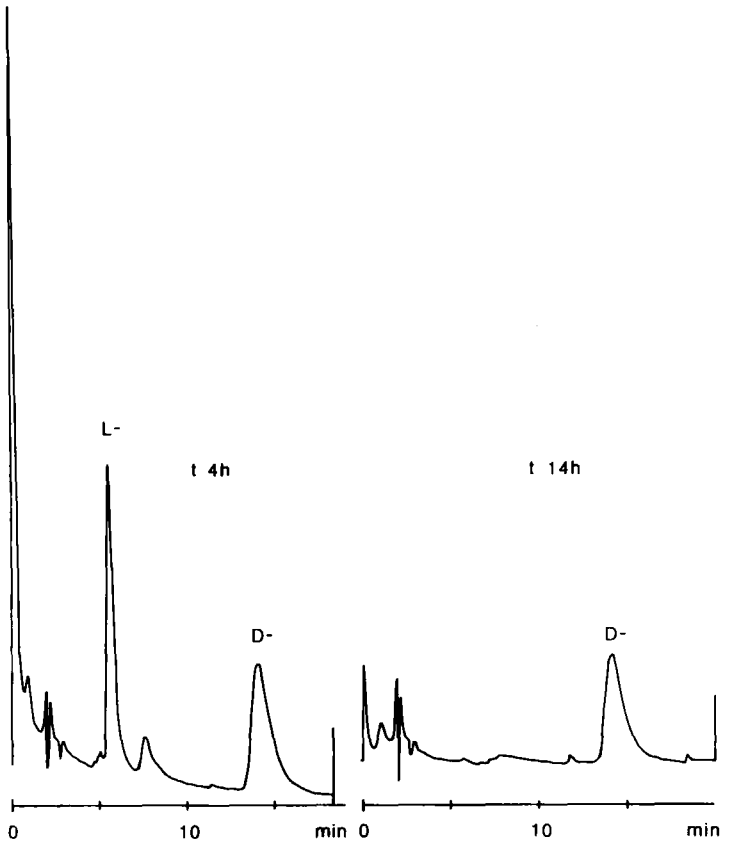


FIGURE 3. Preferential Degradation of N-Benzoyl-L-alanine by Nocardia restrictus as Shown by the Disappearance of the First Chromatographic Peak. The small peak at t_R =7,5 min in the left chromatogram is caused by benzoic acid.

The use of an aqueous mobile phase in HPLC has the further advantage of being compatible with electrochemical detector systems, which in many analytical applications will considerably increase both sensitivity and selectivity. This technique was used in the analytical separation of the enantiomers of 5-hydroxy-D,L-tryptophan as well as 3-hydroxy-D,L-kynurenine on BSA-agarose (24) and will permit the determination of enantiomeric composition in samples of submicromolar concentration.

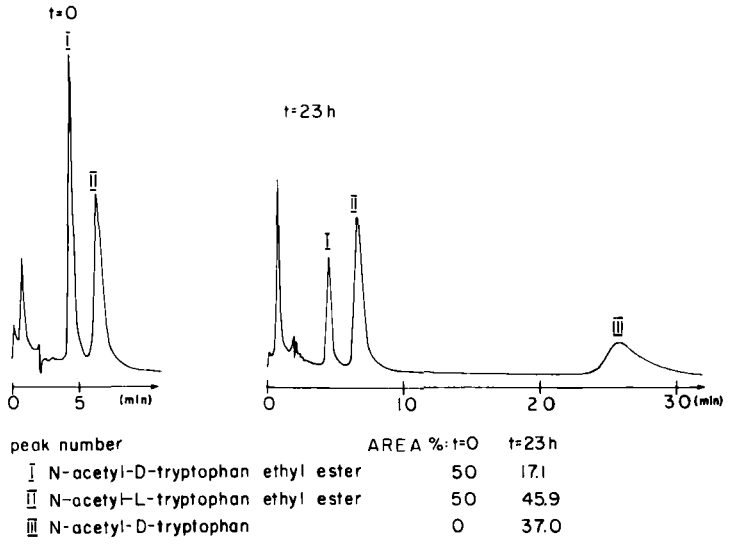


FIGURE 4. Enantioselective Hydrolysis of N-Acetyl-D, L-tryptophan Ethyl Ester by Arthrobacter oxydans. The formation of N-acetyl-D- tryptophan after 23 h. is evident. (Reprinted from ref. 36 with permission).

CONCLUSIONS

The usefulness of BSA as a chemically bonded CSP has been demonstrated by the analytical scale optical resolution of a wide veriety of racemic organic compounds. Very large changes in k′- and α-values can be produced by changes in the mobile phase composition. High k′-values are reduced by the addition of 1-propanol to the mobile phase, indicating the importance of hydrophobic interaction for the over-all retention. Further, in a series of substituted compounds, k′-values tend to increase with the hydrophobicity of the substituent.

The pH of the mobile phase affects the CSP structure as well as protolytic solutes. The largest effects are therefore found on k′- and α-values of weak acids or bases (carboxylic

acids, amines, amino acids). The contribution of electrostatic interactions to over-all retention is also evident from the pronounced effects produced by changes in the ionic strength of the buffer.

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