



## Retention mechanism for ion-pair chromatography with chaotropic reagents

T. Cecchi<sup>a,\*</sup>, P. Passamonti<sup>b</sup>

<sup>a</sup> ITIS Montani, Via Montani 7, 63023 Fermo (AP), Italy

<sup>b</sup> Università degli Studi di Camerino, Dipartimento di Scienze Chimiche, Via S. Agostino 1, 62032 Camerino, Italy

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### ABSTRACT

During the last decade, the extensive use of ion-pair chromatography (IPC) in protein, peptides and basic drugs applications prompted chromatographers to evaluate new additives, since traditional ion-pairing reagents (IPRs) are not usually compatible with LC–MS hyphenation and tend to stick very strongly to the stationary phase, thereby impairing the initial column properties. Chaotropic salts received a great share of credit as tentative IPRs, since they proved to be able to mimic the role of classical IPRs, thereby increasing the retention of oppositely charged analytes. Very few quantitative theoretical studies faced the retention modelling when chaotropic additives are made use of in a chromatographic system and, unfortunately, they used a stoichiometric approach. We hereby debate the present state of the theory and illustrate the first attempt to explain the retention mechanism in the presence of chaotropic reagents in RP-HPLC at a thermodynamic level. We quantitatively validate this model for typical positively and negatively charged analytes as well as for ionic liquid, zwitterionic and neutral analytes.

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### 1. Introduction

Prediction and rationalization of analyte retention, optimization of chromatographic resolution, and simplification of method development call for an adequate theoretical account of RP-HPLC retention process. Eluent composition is by far the most important chromatographic parameter and the mobile phase modification via the use of different additives to improve the chromatographic performance has been keenly and enthusiastically pursued for decades. Particularly, the presence of long chain organic ions in the eluent was demonstrated to be able to enhance the poor RP retention of ionic solutes. A large assortment of names can be found in the literature to mention this separation strategy: [1–11], thereby emphasizing a specific condition that affects retention in the chromatographic system. In the following, the term “ion-pair chromatography” will be used since it received a greater share of credit, even if it was demonstrated that the simple formation of ion-pairs in the eluent does not increase but decreases analyte retention [8].

The success of the optimization procedure may become an intricate task in ion-pair chromatography (IPC), as witnessed by the substantial number of publications that have been devoted to the application of different retention mechanisms to the description of factors affecting analyte retention. Retention modelling has been

both theoretical and empirical. Theoretical models had to face an expanded optimization parameter space (pH, type, lipophilicity and concentration of the ion-pairing reagent (IPR), organic modifier percentage, ionic strength) to predict retention as a function of mobile phase composition, on the basis of fundamental physical principles. The retention mechanism of IPC has been hotly debated for several decades. During the infancy of the theoretical description of IPC, the physical–chemical phenomenon, the basis for the retention mechanism, was alternatively interpreted as ion-pairing or dynamic ion-exchange. The ion-pair model [1,3,12] takes the view that solute ions form neutral ion-pairs with the lipophilic counterions in the mobile phase. These neutral ion-pairs are retained at the non-polar stationary phase with enhanced retention. In contrast, the dynamic ion-exchange model postulates [2,13,14] that the lipophilic ions first adsorb at the surface of the stationary phase, thereby providing exchange sites for analyte ions. Both descriptions lack an unequivocal experimental basis, since, as pointed out by Knox and Hartwick, they are thermodynamically indistinguishable: in fact the initial and final states are the same and the two mechanisms differ only in the sequence of steps, that is unfortunately trivial when dealing with thermodynamic state functions. Actually, these models lead to the same equation to describe retention as a function of the IPR concentration in the eluent [8]. Nevertheless, these models pioneered the stoichiometric approach to the theoretical description of IPC that was followed for decades [15–17] since it does not need a sophisticated mathematical description of analyte retention. It was argued that both ion-pairing and dynamic ion-exchange occur and that the extent to which one is

\* Corresponding author. Tel.: +39 734622632; fax: +39 734622912.  
E-mail address: [teresacecchi@tiscalinet.it](mailto:teresacecchi@tiscalinet.it) (T. Cecchi).

more significant than the other depends on the experimental conditions [18]. Bidlingmeyer and co-workers [4,19] was the first to emphasize the role of the electrical double layer that develops when the IPR dynamically adsorbs onto the RP packing, forming a primary charged ion layer with corresponding counter ions in the diffuse outer region. The retention of the sample involves its transfer through the electrical double layer, hence it results from both electrical and van der Waals forces by means of a mixed retention mechanism. His milestone work questions the epistemological validity of all stoichiometric models because stoichiometric constants, concerning the phase transfer of a charged analyte onto an electrified stationary phase, are not actually constant. On the converse, the equilibrium concentration ratio is potential-modulated; stoichiometric relationships can only describe a physical system fairly well when long-range forces such as coulombic interactions are missing, that is not the IPC case [10,20].

Thermodynamic models share their taking into account the importance of the potential difference between the stationary phase and the bulk eluent that develops because of the different adsorbophilicity of the IPR and its counterion.

Within non-stoichiometric theories there are two models, one by Bartha and Stahlberg [10] and the other by Cantwell [11] that are merely electrostatic since they neglect the ion-pairing process in the bulk eluent. If the principal weakness of stoichiometric models is to neglect the demonstrated development of the electrical double layer, the main shortcoming of these electrostatic models is to ignore the experimental proof [21–27] of the formation of chemical complexes between oppositely charged analyte and IPR. The experimental evidence clearly a-priori impairs and prejudices the theoretical basis of both the purely electrostatic models [10,11]. Actually, these retention mechanisms were demonstrated not to be able to explain a great deal of experimental evidence [20].

Cecchi et al. put forward [20] an extended thermodynamic theoretical treatment of the retention behaviour that takes into account the findings of both stoichiometric and electrostatic models but surpasses them. It exploits the importance of the complex formation at a thermodynamic, and not stoichiometric level, and takes into account the importance of the double layer formation. A tutorial description of the model can be found elsewhere [20].

At variance with the theoretical descriptions, empirical models [28–33] do not need any theoretical basis, and an equation is simply fit to the data; the chemometric approach can be valid and helpful, but does not compare, from an epistemological point of view, to the theoretical approach since the latter dares to convert raw data to knowledge of fundamental principles.

IPC is now an experienced separation strategy. During the last decade, the extensive use of this separation strategy in protein, peptides and pharmaceutical applications prompted chromatographers to evaluate new additives, since traditional IPRs (long chain organic nitrogen cations and long chain sulfates and sulfonates) tend to stick very strongly to the stationary phase. When their presence in the eluent is discontinued, the initial column properties were often found to be impaired. Actually, to effectively wash the column, the presence of an inorganic salt in the aqueous-organic eluent was found to be necessary to provide counterions that may help these organic ions desorption [34]. Furthermore typical IPRs are usually not compatible with LC–MS.

Among newly introduced IPRs, perfluorinated carboxylic acids [35–40] were extensively used in the separations of peptide, amino acids, hydrophilic metabolites and ionogenic bases. They are particularly useful for successful coupling to MS. Their volatility was also valuable when detection was achieved via the evaporative light scattering detector. Their structural similarity with classical IPRs lets model makers suppose that the retention mechanism would

not be very different, except their addition to the mobile phase affects the retention also changing the pH of the eluent.

Polarizable chaotropic ions arranged in the Hofmeister scale are interesting novel IPRs for the analysis of basic analytes [41–58]; they proved to be useful to achieve enhanced retention as well as good resolution, selectivity, and efficiency. Interestingly also weakly chaotropic ions such as those coming from acetic or phosphoric acid can be used as IPRs [42,44].

The rank of an ion in the Hofmeister series is a measure of its chaotropicity, that is related to its charge delocalization and polarizability. Chaotropes ions are not strongly hydrated, hence the water structure is less well-organized. The Greek etymology of the word “chaos” recalls this disordered environment. We have the following sequence in order of increasing chaotropicity [47,50,52]:  $\text{H}_2\text{PO}_4^- < \text{HCOO}^- < \text{CH}_3\text{SO}_3^- < \text{Cl}^- < \text{NO}_3^- < \text{CF}_3\text{COO}^- < \text{BF}_4^- < \text{ClO}_4^- < \text{PF}_6^-$ . Analyte retention is stronger as more chaotropic counterions are used as IPRs [50]. Chaotropic salts have been receiving a growing share of credit as tentative new IPRs, since they proved to be able to mimic the role of classical IPRs with fewer drawbacks. The applications of chaotropic IPC are exponentially increasing; is theory keeping up the pace of practical advances in IPC? Are there any retention mechanism differences between classical IPC and chaotropic IPC? The chaotropic effect challenges model makers. Few quantitative theoretical studies [21,45] faced the retention modelling when chaotropic additives are made use of in a chromatographic system. We hereby critically discuss them and present the first attempt to explain the retention mechanism in chaotropic IPC at a thermodynamic and not only stoichiometric level.

## 2. Theory

Unfortunately, it seems that the theoretical approaches followed to rationalise the retention increase upon addition of chaotropic salts is fraught with the same oversights and omissions that were typical of early retention models of IPC. In fact, the effect of chaotropic additives has been explained following a stoichiometric approach. Many authors advocated for the dominance of the ion-pairing in solution because chaotropic ions are not very hydrophobic. Ion-pairing reactions require the exclusion of water molecules between the pairing charged species. Since chaotropic salts are able to dehydrate more easily compared to kosmotropes, they give strong pairing interactions that allow increased interaction of the neutral complex with the hydrophobic stationary phase. This view was shared by different research groups [42,44,52,53,55,56,58] and was supported by the following experimental evidence: the lower the hydrophobicity of the analyte, the lower the influence of the increase of chaotropic anion concentration on analyte retention [42,52].

A theory was developed by Kazakevich and co-workers [58] to describe the chaotropic effect in the following terms. The retention increase with increase of chaotropic ion concentration was attributed to the degree of analyte solvation: the lower is organic ionic analyte solvation the lower its retention. Since the added chaotropic additives disrupt the solvation shell via ion-pairing, the analyte hydrophobicity improves; this, in turn, leads to enhanced RP retention. The analyte solvation–desolvation equilibrium is described by an equilibrium constant that is tantamount to the ion-pairing equilibrium constant since the desolvated analyte exists only as ion-associated complex with the chaotropic additive. If this equilibrium constant is expressed as a function of the fraction of solvated analyte ( $\theta$ ), it can be easily demonstrated that  $\theta$  increases with decreasing the ion-pairing constant. Now the model takes for granted that the overall retention factor of injected analyte is a sum of the retention factor of the free solvated analyte multiplied by

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