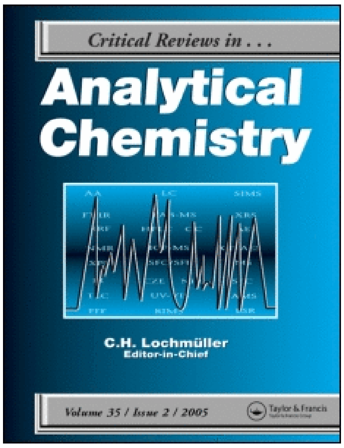

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Ion Pairing Chromatography

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The present state of ion-pair chromatography (IPC) is reviewed. Emphasis is placed on the theoretical modeling of the retention behavior of charged, zwitterionic, and neutral solutes, since the theory provides the generally good understanding of the factors affecting the separation. Although stoichiometric models were credited with the first easy-to-understand demonstration of the retention mechanism of IPC, they were thermodynamic models, consistent with fundamental physics, that extensively detailed the multiplicity of interactions involved in an IPC system. This review is also aimed at giving a broad outline of the progression of the application of this technique during the past decade. Many examples will be given of the analysis of a wide variety of materials, including chiral compounds. They assess the practical potential of this separation strategy. Detection and hyphenation are also described. The relation of IPC to other instrumental method of analysis, the importance of ion-pairing in different (non-extractive) analytical techniques, and not separative aims of IPC, that are topics for which there is a dearth in the literature, are critically reviewed.

Keywords Ion-pairing, retention modelling, experimental factors, detection, applications, hyphenation, comparison, non-separative aims

INTRODUCTION

The analytical strategy that uses lipophilic ions to perform the separation of organic and inorganic ionic solutes with adequate retention, good efficiency and resolution on traditional reversed phase packing is usually referred to as ion-pair chromatography (IPC); the mobile phase additive is usually termed ion pairing reagent (IPR). The eluent pH is a key factor to control analyte charge status and selectivity may be manipulated based basically on both IPR concentration and organic modifier percentage in the eluent. The mechanism of IPC has been a matter of considerable debate during the last three decades. The mechanistic uncertainty is reflected by the large number of alternative terms that are found in the literature for this technique, according to the underscored retention mechanism or chromatographic practice. It was called soap-chromatography (1), solvent generated ion-exchange (2), hetaeric chromatography (3), ion-interaction chromatography (4), hydrophobic chromatography with dynamically coated stationary phase (5), dynamic-complex-exchange chromatography (6), ion-pair adsorption (7) and dynamically coated ion-interaction chromatography (8).

Since it was demonstrated (9) that the simple formation of ion-pairs in the eluent does not increase but decreases analyte retention, the expression "ion-pair chromatography" seems not to be appropriate. Nevertheless, the term IPC has been receiving a greater share of credit also from model makers whose retention models do not postulate the formation of any ion-pair (10, 11).

The essence of this separation mode consists in the dynamic modification of the surface of a reversed-phase (RP) chromatographic packing by the adsorption of hydrophobic ions. The pioneering work by Bidlingmeyer et al. (4) highlighted how different the actual retention mechanism of this separation strategy is from a simple stoichiometric ion-pairing process. They qualitatively demonstrated that a complex ion-interaction mechanism qualitatively explains analyte retention. In a series of papers, Cecchi et al. (12–26) put forward an extended physical chemical theoretical treatment of the retention behavior that quantitatively confirms that the term ion interaction chromatography is the most correct one. Nevertheless, in the present review, the common term IPC will be used for the sake of clarity.

IPC is a mature and valuable separation strategy. Its scope is considerable, with a very wide range of reported applications concerning organic and inorganic ions, neutrals and zwitterionic compounds. New or modified approaches (ultra-fast separation, hyphenation, use of the normal phase, biotechnological and not separative aims) are introduced very rapidly. In this context it is important to underline that a trade-off between breadth and depth is obviously necessary.

We attempted to cover most developments that increased the scope of IPC during the last decade.

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RETENTION MODELLING

Raw retention data in IPC would be meaningless without some form of analysis to gain knowledge. A model is a representation of reality. Basically, there are two types of models: theoretical and empirical. Theoretical models apply known theoretical laws or principles to convert raw data to knowledge. New data follow from the theory that is predictive in its own right. On the converse, empirical models do not need any theoretical basis, and an equation is simply fit to the data: the response is regressed onto a set of descriptors and one must assume that the observed trend continues outside the experimental range. A major challenge in the development of chromatographic procedures is a rational and judicious selection of optimal experimental conditions that can provide adequate resolution and reasonable run time. The success of the optimization process may become a non-trivial task in IPC of complex samples that contain non-ionic, ionizable and ionic solutes, owing to an expanded optimization parameter space (pH, type and lipophilicity of the IPR, organic modifier and IPR concentration, ionic strength, stationary phase packing) and the wide differences in the analyte nature. In the early stage of the IPC practice, prediction of given analyte retention was commonly faced with trial-and-error procedures that attempted to make the problem univariate, holding all experimental conditions constant except one. Later, modelling of the dependence of retention on the mobile phase composition was the focus of interest of many chromatographers since an a priori retention prediction was highly desirable. More recently the dependence of retention on the analyte nature was faced via the use of quantitative structure retention relationships that aim at correlating k with molecular descriptors.

Modelling of Retention as a Function of the Mobile Phase Composition

Theoretical Models of IPC

This section critically evaluates the currently available theories of this separation mode. Non-stoichiometric models are described in more detail because they are credited with firm foundations in physical chemistry.

Stoichiometric Models. Stoichiometric models are pictorial and, since they do not need a sophisticate, mathematical description of analyte retention, they pioneered the rationalization of the retention patterns in IPC. Stoichiometric models are a range of retention mechanisms. The reaction scheme upon which they are built may be different, but since all of them suggest the same structure of the adsorbed complex, they lead to identical retention equations (9). In this context it is worth underlining that the IPC expression derives from the historical application of the ion-pair concept (27). According to the classical ion-pair model (1, 3, 28) the IPR and the solute ion combine in the eluent to form a neutral species which is then able to partition into the hydrophobic stationary phase. In contrast, the dynamic ion-exchange model postulates (2, 29, 30) that the lipophilic ions first adsorb at the surface of the stationary phase, and these dynamically generated charge sites serve as exchange sites for analyte ions. Retention of lipophilic ions on the stationary phase has been the major supporting evidence for the ion-exchange mechanism even if this model is not able to explain the contribution of solute hydrophobicity to retention since selectivity arise only from differences in electrostatic interactions. It can be argued that both mechanisms occur and that the extent to which one is more significant than the other depends on the experimental conditions (31). Other stoichiometric models are based on various combinations of the above-described ion-pair and dynamic ion exchange mechanisms: mixed modes, the displacement of the IPR by the ion pair complex (3), the effect of the IPR counter i on (32), and the reduction of the available hydrophobic surfaces of the stationary phase (33) were all considered responsible for different retention patterns.

It is, however, important to underline that not even the most recent stoichiometric comprehensive models (34, 35) can provide a true description of the real retention mechanism because stoichiometric constants are not actually constant under the long-range electrostatic interactions that characterize the IPC system (10, 12). According to the retention model of Bidlingmeyer (4, 36), which is broader in scope than either a dynamic ion-exchange mechanism or an ion-pair mechanism the lipophilic IPR flows under isocratic conditions, since it is included in the eluent, and it dynamically adsorbs onto the alkyl-bonded apolar surface of the stationary phase, forming a primary charged ion layer. The corresponding counter ions are found in the diffuse outer region to form an electrical double layer. The retention of the sample involves its transfer trough the electrical double layer, hence it results from both electrical and van der Waals forces by means of a mixed retention mechanism. These observations paved the way to the development of non-stoichiometric models of IPC and non-consistency of stoichiometric models with fundamental physics entitles the present author not to describe stoichiometric models in more depth.

Non-Stoichiometric Models. All non-stoichiometric models share their taking into account the importance of the electrical double layer and the potential difference between the stationary phase and the bulk eluent due to the different adsorbophilicity of the IPR and its counterion. Some attempts have been made by other research groups (37–39) to formulate the ion-interaction model by Bidlingmeyer et al. (4, 36) quantitatively, but they do not provide a rigorous description of the system.

Within non-stoichiometric theories there are two models, one by Stahlberg et al. and the other by Cantwell et al., that are purely electrostatic: they consider the processes involved in IPC as based solely on the formation of the electrical double layer and disregard the ion-pairing process in the bulk eluent.

In the electrostatic model by Stahlberg et al. the description of the double layer is based on the Gouy-Chapman (G-C) theory that gives the electrostatic potential profile as a function of the distance from the surface. The primary charged layer is due to the adsorption of the IPR, the secondary diffuse layer is due to the IPR counterions. Both layers are under dynamic equilibrium.

The adsorbed IPR ions are responsible for the electrical potential difference, Ψ°, between the surface and the bulk solution. Since counter ions of opposite signs, are electrostatically attracted toward the charged surface, like sign co-ions are repelled from the surface and the potential decays almost exponentially because of screening effects.

In the electrostatic model by Bartha and Stahlberg, reviewed in ref. (10), solute retention in IPC is governed by the free energy of adsorption of the analyte $\Delta G^\circ_{t,E}$. There are two contributions to $\Delta G^\circ_{t,E}$: the first one is the chemical part $\Delta G^\circ_{c,E}$ that is a measure of the analyte hydrophobicity and corresponds to the free energy of adsorption of the analyte in the absence of the IPR; the model assumes that it remains constant in the presence of the IPR. The second one, $\Delta G^\circ_{el,E,}$ is the electrostatic contribution that represents the work involved in the transfer of a charged analyte to the charged stationary phase. It can be easily demonstrated that $\Delta G^\circ_{el,E}$ is related to the surface electrostatic potential Ψ° via the following relationship:

$$\Delta G^\circ_{el,E} = z_E F \Psi^\circ \qquad [1]$$

where z_E is the analyte charge and F is the Faraday constant; it follows that

$$\Delta G^\circ_{t,E} = \Delta G^\circ_{c,E} + z_E F \Psi^\circ \qquad [2]$$

Since the model does not take into account the possibility that the analyte adsorbs as an ion-pair from basic chromatographic theory, the following relationships hold:

$$K_{t,E} = \exp(-\Delta G^\circ{}_{t,E}/RT) \qquad [3]$$

$$k = \phi \frac{[LE]}{[E]} = \phi K_{t,E} = \phi .\exp\left(-\frac{\Delta G^0_E}{RT} - \frac{z_E f \Psi^0}{RT}\right) = k_0 \exp\left(-\frac{z_E F \Psi^0}{RT}\right) \qquad [4]$$

where ϕ is the phase ratio of the column, (LE) and (E) are, respectively, the surface and the mobile phase concentration of the analyte E, $K_{t,E}$ is the thermodynamic equilibrium constant for the adsorption of the analyte E onto the stationary phase, k_0 is the capacity factor of the analyte in the absence of the IPR, that is when the surface potential is zero, T is the absolute temperature, and R is the gas constant. To obtain an expression between the analyte capacity factor and mobile phase concentration of IPR for practical test, a series of approximations have to be made (10). Firstly, the surface potential given by the linearized Poisson expression was used, even if the surface potential can easily exceed the threshold limit (25 mV) above which the simplified expression does not hold anymore. The surface concentration of the IPR is given by the potential modified Langmuir adsorption isotherm but the linearization of the adsorption isotherm (necessary to avoid a too complex expression) introduces a further approximation in the retention model. Unfortunately, since the stationary phase coverage can be high even at very low eluent concentrations, the linearization is not usually acceptable under typical experimental conditions in IPC (12, 13, 40, 41). Recently, a method that helps model makers interested in ionic interactions determine whether the potential modified Langmuir isotherm can be linearized and, accordingly, whether simplified retention equations can be properly used was developed (41). Moreover, a surface potential empirically obtained from retention data is also used (at variance with reality, different apparent surface potentials are obtained when different probe solutes are used (13)) and a series expansion of a double logarithmic term is also necessary. It has to be underlined, however, that the major concern that arises upon analyzing the electrostatic theory of IPC is not the series of approximations or the empirical "contamination" of the theoretical model, but exactly the proposed mechanism that does not take into account the formation of ion-pairs. In the following we will highlight that since this retention model ignores the presence of ion-pairs, it is not able to explain large experimental evidence, not even if a multi-site occupancy model (42) is considered.

The second electrostatic model, put forward by Cantwell et al. and reviewed in ref. (43), from both a conceptual and a practical point of view, is more complex than the previous. It is based on both ion-exchange and interaction with the electrical double layer (44–46). The description of the electrical double layer is based on the Stern-Gouy-Chapman (SGC) version of the theory (47). It takes into account that counter ions are not able to get closer to the surface than a certain distance (plane of closest approach of counter ions). Not chemically adsorbed ions are located in the Outer Helmholtz Plane (OHP). It is situated at a distance x_o from the surface. The potential difference between this plane and bulk solution is Ψ_{OHP}. In this version of the theory Ψ_{OHP}replaces Ψ° in all equations. Two regions are distinguishable in the double layer: the compact part between the charged surface and the OHP, in which the potential decays linearly, and the diffuse layer in which the potential decays almost exponentially because of screening effects. The higher the ionic strength, the faster the potential decays.

The experimental results by Cantwell et al. suggest that the main process that determines retention is ion exchange of the solute ion between the bulk of the mobile phase and the diffuse part of the diffuse layer. To use the model, a family of adsorption isotherms of the IPR (each isotherm is measured at constant eluent ionic strength) and retention data of the analyte for the same set of IPR and electrolyte concentrations are needed; hence, testing and application of the model is not straightforward. As in the model by Stahlberg et al., it neglects the formation of ion-pairs; hence, the same major criticism applies also to this theory.

In this context it is worth emphasizing that all retention models have had their disciples and were experimentally confirmed, but a simple fit of experimental results does not imply that the theory is correct. To be acceptable a theory that explains retention in the chromatographic system must be well founded in physical chemistry and must describe all experimental evidence.

If the paramount drawback of stoichiometric models is to neglect the demonstrated development of the electrical double layer, the main problem of electrostatic models is to ignore the experimental proof of the formation of chemical complexes between oppositely charged analyte and IPR (48–51). Ion-pairing reactions belong to equilibria that can be examined with the aid of an electrophoretic method: it was observed that the electrophoretic mobility of large anions decreased with increasing concentrations of the IPR and the ion association constants can be estimated by analyzing the shift of the analyte peak after the addition of the IPR in BGE background electrolyte (BGE) (52–55). In an attempt to replace the chromatographic hydrophobic interaction mechanism located at the interface with a hydrophobicity ion-pairing based mechanism located in the capillary electrophoresis (CE) BGE, Popa et al. deeply studied ion pairing in the BGE in the CE separation of diastereomeric peptide pairs (56): in spite of the absence of a hydrophobic surface, a clear separation according to the analyte hydrophobicity was observed. Since it improves with increasing IPR concentrations and hydrophobicity, the CE method achieves a bi-dimensional separation according to the analyte charge and hydrophobicity, thereby demonstrating the ion-pairing process in solution (57). It was also more interesting to observe that hydrophobic ion-pairing equilibria could not be explained by a purely electrostatic approach (21, 58).

All these experimental evidences clearly a-priori impair and prejudice the theoretical basis of both the purely electrostatic models by Stahlberg et al. and by Cantwell et al.

An attempt to face molecular interactions between solutes, IPR and solvents was put forth by Lu et al. (59): they used a statistical thermodynamic method, combined with the Freundlich isotherm, to obtain a retention equation as a function of IPC concentration, ionic strength, and organic modifier percentage in the eluent. They took into account both electrostatic and molecular interactions to obtain the chemical potential of different species. Equilibrium conditions require that both chemical potentials of a species in the mobile and in the stationary phase should be equal. The description of electrostatic and pairing interactions at a molecular level is very complicated. For example, the chemical potential of the IPR in the adsorbed phase depends (i) on the internal partition functions of the solvents and of the IPR itself and (ii) on the potential energy of the solvents and of the IPR itself on the stationary phase; the latter, in turn, depends, among others, on constants related to the molecular size of both the IPR and of each solvent and the magnitude of the molecular interaction of the IPR and each solvent. For the solute ion the expression for the chemical potential is still more complicated. Their retention equations fit experimental results but the method they used resulted in a large number of fitting parameters whose physical meaning is clear even if complicated. Their estimates are impossible to be commented upon, since many of the cited parameters are not known. The author concluded that a more detailed investigation of the effects of the structure and physical chemical parameters of the solute on the coefficient of the fitted equations was necessary, but they did not continue their work.

The thermodynamic constants actually are related to all the molecular level interactions taken into account by the model by Lu et al., but describe in a very simple way the studied chromatographic system and, primarily, can be related to their non-chromatographic estimates to confer a spare validation to the theoretical model. Starting from this idea, in a series of papers, Cecchi et al. put forward an extended physical chemical theoretical treatment of the retention behavior that encompasses and comprehends both stoichiometric and electrostatic models but surpasses them. The model is evidence-based and well founded in thermodynamics. It exploits the importance of the complex formation at a thermodynamic, and not stoichiometric, level and takes into account the stationary phase electrostatic potential that results from the IPR adsorption. The issue is not complicated and a tutorial method for approaching it will be given below. A more detailed and comprehensive description of the model can be found elsewhere (12, 60).

The electrical potential difference (Ψ°) that develops between the surface and the bulk solution is given by the rigorous G-C theory equation, even if for high (15, 17, 19) or low (16, 20) surface potential the rigorous expression can be approximated.

The Freundlich adsorption equation is related to the potential modified Langmuir adsorption isotherm that holds for the adsorption of ions which have a substantial hydrophobic moiety, as IPRs, hence it is not empirical (61); the limit between a theoretical and an empirical use of the Freundlich isotherm was recently established (41). The surface potential established by the adsorption of the IPR always runs counter to further adsorption of the IPR since Ψ° is of the same sign as the charge of IPR (z_H). The Freundlich adsorption isotherm was used to describe the surface concentration of the IPR (LH) as a function of its mobile phase concentration (H):

$$[\mathrm{LH}] = \mathrm{a}[\mathrm{H}]^{\mathrm{b}} \qquad [5]$$

where *a* and*b*are two constants that can be experimentally evaluated.

In the chromatographic system the main equilibria are the adsorption of the analyte E onto the stationary phase hydrocarbonaceous ligand site L, the adsorption of H onto L, the ion-pair formation in the mobile phase (EH), the ion-pair formation in the stationary phase (EHL), and the displacement of H by E. The thermodynamic equilibrium constants for these equilibria are, respectively, K_{EL}, K_{HL}, K_{EH}, K_{EHL}, and K_{EL}/K_{HL}.

In the following they will be derived. If the charge status of the analyte and IPR are the same, or if the analyte is neutral, ion-pairing equilibria do not apply. For the condition of equilibrium it holds that:

$$\mu_{\mathrm{LE}} = \mu_{\mathrm{L}} + \mu_{\mathrm{E}} \qquad [6]$$

$$\mu_{\mathrm{LH}} = \mu_{\mathrm{L}} + \mu_{\mathrm{H}} \qquad [7]$$

$$\mu_{\mathrm{EHL}} = \mu_{\mathrm{E}} + \mu_{\mathrm{H}} + \mu_{\mathrm{L}} \qquad [8]$$

$$\mu_{\mathrm{EH}} = \mu_{\mathrm{E}} + \mu_{\mathrm{H}} \qquad [9]$$

where μ represents the electrochemical potential for each species that takes into account the development of the surface potential for adsorbed charged species. For example, we have:

$$\mu_{\mathrm{LE}} = \mu^{\circ}_{\mathrm{LE}} + \mathrm{R}T \ln a_{\mathrm{LE}} + z_{\mathrm{E}}\mathrm{F}\Psi^{\circ} \qquad [10]$$

where μ° represents the electrochemical potential for the standard state. Similar expressions can be obtained for the electrostatic potentials of each other species.

Since a thermodynamic equilibrium constant has the general expression

$$\mathrm{K} = \exp(-\Delta\mu^{\circ}/\mathrm{RT}) \qquad [11]$$

the following equations are easily obtained for each thermodynamic equilibrium constants (a represents the activity of each species):

$$K_{\mathrm{LE}} = \frac{a_{\mathrm{LE}}}{a_{\mathrm{L}} \cdot a_{\mathrm{E}}} \exp(z_{\mathrm{E}}\mathrm{F}\Psi^{\circ}/\mathrm{RT}) \qquad [12]$$

$$K_{\mathrm{LH}} = \frac{a_{\mathrm{LH}}}{a_{\mathrm{L}} \cdot a_{\mathrm{H}}} \exp(z_{\mathrm{H}}\mathrm{F}\Psi^{\circ}/\mathrm{R}T) \qquad [13]$$

$$K_{\mathrm{EH}} = \frac{a_{\mathrm{EH}}}{a_{\mathrm{E}} \cdot a_{\mathrm{H}}} \qquad [14]$$

$$K_{\mathrm{EHL}} = \frac{a_{\mathrm{EHL}}}{a_{\mathrm{E}} \cdot a_{\mathrm{H}} \cdot a_{\mathrm{L}}} \qquad [15]$$

Equations [12] and [13] parallel the non-thermodynamic, stoichiometric (or concentration based) constants of stoichiometric models (see Eqs. [1] and [3] and [9]), which neglect the importance of the electrostatic potential: it follows that a stoichiometric constant is not actually constant (10) after the addition of the IPR in the mobile phase, since it alters the surface potential, which in turn modulates the influence of increasing IPR concentration.

If ion-pairing equilibria are taken into account, the capacity factor of the analyte, k, is

$$k = \phi \frac{[\mathrm{LE}] + [\mathrm{EHL}]}{[\mathrm{E}] + [\mathrm{EH}]} \qquad [16]$$

where [EHL] and [EH] are, respectively, the surface and mobile phase concentration of the complex EH. It can be easily demonstrated that (12) the course of analyte retention upon the mobile and stationary phase concentration of the IPR can be described, under trace conditions (that is if the IPR concentration is much higher than that one of the analyte), by the following expression:

$$k = \phi[L]_T \times \frac{K_{\mathrm{LE}}\frac{\gamma_{\mathrm{L}}\gamma_{\mathrm{E}}}{\gamma_{\mathrm{LE}}} \exp(-z_{\mathrm{E}}\mathrm{F}\Psi^{\circ}/\mathrm{R}T) + K_{\mathrm{EHL}}\frac{\gamma_{\mathrm{E}}\gamma_{H}\gamma_{\mathrm{L}}}{\gamma_{\mathrm{EHL}}}[\mathrm{H}]}{\left(1 + K_{\mathrm{EH}}\frac{\gamma_{\mathrm{E}}\gamma_{\mathrm{H}}}{\gamma_{\mathrm{EH}}}[\mathrm{H}]\right)\left(1 + K_{\mathrm{LH}}\frac{\gamma_{\mathrm{L}}\gamma_{\mathrm{H}}}{\gamma_{\mathrm{LH}}} \exp(-z_{\mathrm{H}}\mathrm{F}\Psi^{\circ}/\mathrm{R}T)[\mathrm{H}]\right)} \qquad [17]$$

where $[\mathrm{L}]_{\mathrm{T}}$ estimates the total ligand surface concentration, and γ represents the activity coefficient for each species. A parallel expression can be easily derived for the dependence of the retention as a function of the stationary phase concentration of the IPR.

The first term in the numerator of Eq. (17) describes how the surface potential influences the analyte retention. The interaction would be attractive (repulsive) if the analyte is oppositely (similarly) charged to the IPR; hence, analyte retention would always increase (decrease) with increasing IPR concentration if this were the only mechanism in the system. The second term in the numerator of Eq. [17] accounts for ion-pair formation at the stationary phase: it can be demonstrated that it can result from the simultaneous adsorption of both E and H onto L, or from the adsorption of E onto LH, or from the adsorption of H onto LE, or from the adsorption of EH onto L. These equilibria are not independent of each other, hence the mathematical treatment can be simplified. Ion-pairing at the stationary phase always results in a retention increase. The left factor of the denominator of Eq. [17] accounts for ion-pair formation in the mobile phase: since in this case the analyte is withdrawn from the stationary phase towards the eluent, this interaction reduces retention, at variance with the classical view of a retention increase because of ion-pairing. Both terms concerning ion-pair formation are missing if the analyte and IPR are similarly charged, or if the analyte is neutral. The right-hand factor of the denominator of Eq. [17] describes the competition between the analyte and the IPR for the available ligand sites that decrease with increasing IPR concentration.

When the G-C expression for the potential and the Freundlich expression for the adsorption isotherm of the IPR are used it can be easily demonstrated that the following expression is obtained [12]:

$$\mathrm{k} = \frac{\mathrm{c}_1\left(\mathrm{a}[\mathrm{H}]^{\mathrm{b}}\mathrm{f} + \left((\mathrm{a}[\mathrm{H}]^{\mathrm{b}}\mathrm{f})^2 + 1\right)^{\frac{1}{2}}\right)^{\pm 2|\mathrm{z}_{\mathrm{E}}|} + \mathrm{c}_2[\mathrm{H}]}{(1 + \mathrm{c}_3[\mathrm{H}])\left(1 + \mathrm{c}_4[\mathrm{H}](\mathrm{a}[\mathrm{H}]^{\mathrm{b}}\mathrm{f} + ((\mathrm{a}[\mathrm{H}]^{\mathrm{b}}\mathrm{f})^2 + 1)^{\frac{1}{2}}\right)^{(-2|\mathrm{z}_{\mathrm{H}}|)}\right)} \qquad [18]$$

where:

$$\mathrm{f} = \frac{|\mathrm{z}_{\mathrm{H}}|\mathrm{F}}{\left(8\varepsilon_0\varepsilon_{\mathrm{r}}\mathrm{RT}\sum_{\mathrm{i}}\mathrm{c}_{0\mathrm{i}}\right)^{\frac{1}{2}}} \qquad [19]$$

f (m^2/mol) is a constant which can be evaluated from the eluent composition.

In the exponent of the first term of the numerator, the plus (minus) sign applies for oppositely (similarly) charged analytes and IPR, as expected on the basis of the electrostatic behavior. The ion-pairing terms (c_2, c_3) are absent for similarly charged analytes and IPR. Eq. [18] is also able to take into account, via the magnitude of z_E in the exponents, that the electrostatic interaction is stronger for multiply charged analytes (22).

When Eq. [18] is fitted to experimental data, excellent results are obtained (12–14, 22, 24).

Fitting parameters $c_1 - c_4$ are not simple adjustable constants, but have a clear physical meaning (12) and this allows the parameter estimates to be commented upon. Since c_1 represents k_0 (that is analyte retention without the IPR in the mobile phase) it can be obtained from experimental results, hence it was not considered an optimization parameter. However, when c_1 was estimated by the model, the percent error is very low (0.6%); c_2 and c_3 are related to ion-pair equilibrium constants and increase with increasing analyte lipophilicity; and c_4 represents the equilibrium constant for the IPR adsorption. The values of these parameters, estimated by the model from the fitting of retention data, are very reasonable and agree with chromatographic and non-chromatographic estimates (12–14, 22, 24, 61, 62).This lends strong support to the model.

The retention model by Cecchi et al. is the first one that quantitatively focused its attention on the retention behavior of neutral and zwitterionic analytes in IPC. According to the electrostatic model, the retention of an uncharged solute is not dependent on the presence and concentration of a charged IPR in the chromatographic system but this runs counter several experimental evidence (9, 14, 17). Actually, the retention of neutral analytes was found to slightly decrease with increasing IPR concentration. Eq. [18] simplifies: if z_E is zero it (14) simplifies since ion-pairing does not occur ($c_2 = c_3 = 0$). Adsorption competitions model the retention pattern of neutral analytes in IPC; the experimental evidence of a residual retention decrease can be quantitatively explained (14, 17).

The surface potential is also easily predicted to alter the capacity factor of zwitterionic solutes (18–20, 25, 26) that are characterized by an electrical dipole. In the non-homogeneous electrical field that develops at the interphase the dipole is subjected to a torque moment that arranges it parallel to the lines of the field, with the head oppositely charged to the electrostatic potential facing the stationary phase surface. Hence, the electrical force is always attractive and it pushes the dipole towards the interphase, where the field is stronger. This is tantamount to consider the electrical dipole equivalent to a fractional electrical charge. The force acting on the dipole or on the equivalent fractional charge was calculated and was used to give the electrostatic contribution to the electrochemical potential of the analyte, and hence to the thermodynamic equilibrium constant for its adsorption. Both the dipole and the fractional charge approaches proved to be very effective in describing the retention patterns of zwitterions (18–20, 25–26). Both of them were experimentally validated since they both give the same retention equation, even if the physical meaning of one fitting parameter is different.

$$k = \frac{c_1(a[\mathrm{H}]^b f + ((a[\mathrm{H}]^b f)^2 + 1)^{\frac{1}{2}})^{2c_2 K/\mathrm{F}}}{1 + c_4[\mathrm{H}](a[\mathrm{H}]^b f + ((a[\mathrm{H}]^b f)^2 + 1)^{\frac{1}{2}})^{-2|z_H|}} \qquad [20]$$

In Eq. [20], c_1 and c_4 are the already discussed parameters, while c_2 is related to the molecular dipole (18), or alternatively to the fractional charge (25–26): the higher it is, the stronger is the retention increase upon IPR addition. Equation [20] can be advantageously used in life science chromatography since it is of utmost importance to keep peptides at their isoelectric point to avoid denaturation.

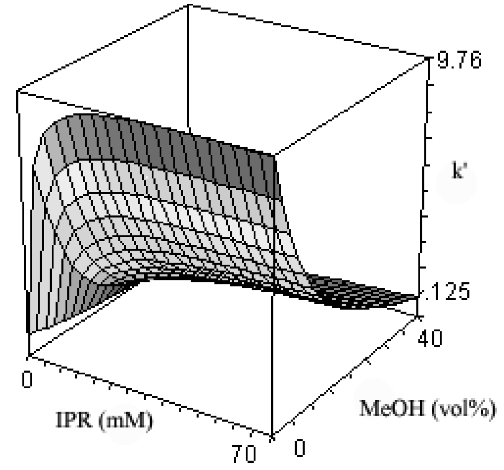

FIG. 1. Retention behavior of a typical analyte in IPC as a function of IPR mobile-phase concentration and organic modifier percentage in the eluent.

The retention model by Cecchi et al. treats the simultaneous influence of both the organic modifier and IPR concentrations in the eluent from a bivariate point of view (21), as illustrated in Figure 1. The retention equation predicts that retention decreases with increasing organic modifier concentration and increases with increasing IPR concentration, as expected. Interestingly, this increase is lower at high organic modifier percentages since the organic modifier reduces analyte retention both directly (it decreases the analyte free energy of adsorption) and indirectly (it decreases the IPR free energy of adsorption on the the stationary phase, hence the electrostatic potential is lower—this in turn results in a lower retention).

In the absence of the IPR, the bivariate retention equation reduces to the expression that describes the influence of the organic modifier on RP-HPLC retention (63).

The retention model by Cecchi et al. also treats the simultaneous influence of both the ionic strength and IPR concentration in the eluent from both monovariate and bivariate approaches (23).

During a typical IPC optimization procedure, when a compensatory electrolyte is not added to the eluent, the ionic strength increases only as a result of increasing H concentration since the background electrolyte concentration is kept constant. The ionic strength influences the surface potential; hence, the dependence of f(Eq. [16]) on the ionic concentration must be explicitly addressed (23). With increasing electrolyte concentration the electrostatic potential decreases since counter-ions in the diffuse layer partially mask the surface charge. Simultaneously there is an increased surface concentration of the IPR because counter-ions lower the self-repulsion forces between the similarly charged adsorbed IPR ions (Donnan effect) (45, 46, 62, 64). This issue interlocks with the former: at higher ionic strength, the higher degree of H adsorption partially compensates for the decrease in magnitude of the electrostatic surface potential. Yet a neat decrease of the surface potential is obtained at higher electrolyte concentrations. The strong interplay between these issues was easily taken into account using the adsorption isotherm of the IPR obtained with changing ionic strength (23). Moreover, the model considers that solutes undergo salting-in or salting-out. According to the solvophobic theory (3), the former (latter) is predicted when the addition of the salt results in a decreased (increased) surface tension of the eluent. Hydrophobic ions as IPRs are usually salting-in agents since they decrease the surface tension of the eluent (3). The solvophobic theory can be used to obtain the dependence of the equilibrium constants on ionic strength. When Eq. [18] is modified according to the issues above the relationship that can be obtained is algebraically quite complex but usually it can be strongly simplified because salting effects are often negligible, at least if the ionic strength increase is not very strong. The retention equation reduces to the one developed and tested in RP-HPLC (65, 66) if H is not present in the eluent and this, again, confirms the strength of this new theoretical model. When both the background electrolyte and the IPR concentrations are varied the retention needs to be described by a bivariate expression (23). Figure 2 details this issue. The relationship predicts that retention of a solute oppositely (similarly) charged to the IPR decreases (increases) with increasing ionic strength, because of the lower net electrostatic attraction (repulsion). The predictions are experimentally verified (23).

In conclusion, the claims for a superior theory, epistemologically speaking, are supported by the following arguments:

- The model is comprehensive: the retention equations quantitatively predict the retention pattern of charged, multiply charged, neutral, and zwitterionic solutes in IPC, and can also quantitatively take into account the influence of the organic modifier concentration and of the ionic strength.
- The model, in the absence of IPR, reduces to the well known relationships of RP-HPLC for the influence of the organic modifier or the ionic strength in the eluent.
- If the surface potential or ion-pairing equilibria are disregarded the model reduces, also from the algebraic point of view, to the relationships of stoichiometric or electrostatic approaches in IPC, respectively, thus the model by Cecchi et al. can be considered to embed the previous retention models but also to master them.
- It is evidence based. It is able to rationalize also: (i) different theoretical curves when k is plotted as a function of the stationary phase concentration of the IPR, for different IPRs; (ii) dependence of the ratio of the retention of two different analytes on the IPR concentration; and (iii) dependence of the k/ko ratio on the analyte nature if the experimental conditions are the same—these experimental behaviors cannot be explained by other outstanding electrostatic retention models because they can only be rationalized by the complex formation.
- Adjustable parameters have a clear physical meaning and their estimates are trusty since they compare well with literature chromatographic and non-chromatographic estimates.
- It is the first model that takes into account at a thermodynamic level the recently quantitatively demonstrated ion-pairing equilibria in the mobile phase.

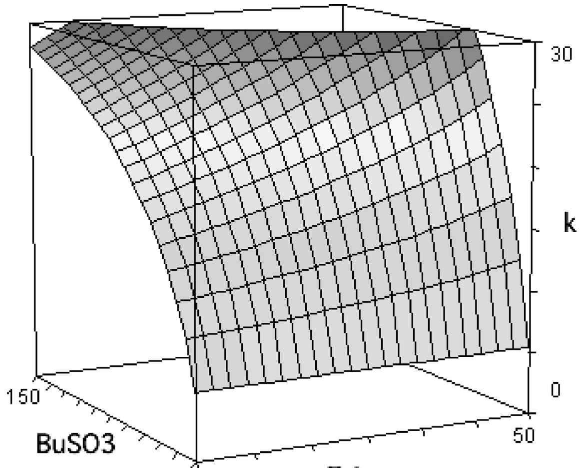

FIG. 2. Retention behavior of a typical analyte in IPC as a function of IPR (butylsulfonate, BuSO3,) mobile-phase concentration (mM) and indifferent electrolyte ionic strength Σc_i (mM) in the eluent.

Empirical Models of IPC

Computer aided chemometrical optimization methods give up to theoretical prediction of the behavior of the system; they were developed because experimental parameters are interlocking and gave too much information to easily take in at once. Experimental design is aimed at letting the chromatographer find the best way to obtain the desired result, but if the starting values of the parameters are not judiciously chosen they often yield only local optima; moreover, predictions out of the studied parameters' ranges are usually not possible. The efficient use of factorial designs for optimum parameter selection requires a large number of chromatographic runs; statistical mixture design, often search only a certain part of the optimization parameter space.

A central composite design for separation of As and Se species in tap water took into account the influence of the ionic strength, IPR concentration and pH; response surfaces and iso-response curves allowed the determination of the optimum chromatographic conditions and of the robustness of the method, coupled with plasma MS detection (67).

A central composite design was also used to evaluate the influence of the organic modifier and of the IPR concentration on retention conditions of biogenic amines in wines. This study constituted the basis for a further optimization, since a gradient elution was needed to obtain reasonable chromatographic times: in the subsequent central composite design the factors to be optimized were the time for the initial isocratic step with

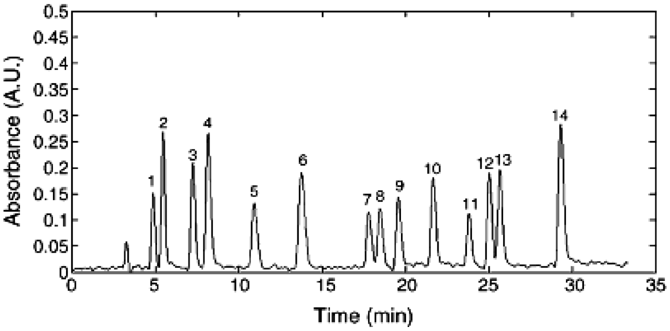

FIG. 3. Chromatogram of a biogenic amine standard mixture using the optimized conditions. Composition of the sample 0.3 mM each amine. Peak assignment: 1 = ethanolamine, 2 = ethylamine, 3 = octopamine, 4 = dopamine, 5 = tyramine, 6 = serotonine, 7 = 1,2-diaminobutane, 8 = histamine, 9 = agmatine, 10 = phenylethylamine, 11 = tryptamine; 12 = cyclooctylamine, 13 = spermidine, 14 = spermine. See ref. (68) for details. Reprinted from ref. (68) with permission from Elsevier.

100% of Eluent A and the time for the linear increase of Eluent B from 0 to 45%, that is related to the gradient steepness. To obtain a trade off between resolution and chromatographic time an optimization through a multicriteria approach based on Derringer desiderability function was followed (68). The resulting chromatogram is shown in Figure 3.

A central composite design (24 + star) with four factors was performed in order to optimize the elution gradient program of the perfusion RP-HPLC method for the characterization of maize products. The experiments were carried out in random order and by triplicate. The resolution between adjacent peaks and the analysis time were selected as six responses for this design. Mathematical models correlating the responses to the four variables and their interactions were calculated using a second order regression and were then employed for calculating the values of the variables that optimize each response. The optimized method allowed the rapid separation of maize protein in 3.4 min (69).

The chromatographic behavior of anionic specimens in ion-pair reversed-phase liquid chromatography was investigated by the method of the three-factor first-order mathemathical modeling. Empirical regression equations were deduced for the dependence of the sorption capacity factor on the concentration of ion-pair reagent, amount of organic modifier, and pH of mobile phase (70).

A full factorial experimental design was also used to collect sound retention data of inorganic anions on a dye-coated stationary phase in order to validate a retention model on the basis of the two main processes in of ion-exchange and ion-pair formation for inorganic anions in IPC (71). A two-levels full factorial design was performed to investigate the effects of the percentage organic modifier and the content of the IPR in the eluent on retention of heterocyclic aromatic amines. A star design was added to the factorial design experiments since some analytes showed relevant quadratic effects. The final regression models were then calculated using the central composite design (CCD) experiments obtained both from the full factorial design and the star design (72).

The prediction power of hard modelling and neural-network models in IPC were compared: the study demonstrated that the former was more accurate when few experimental data were available, while the prediction power was similar for a large number of data. It can be observed that the neural-network software accounted for a greater versatility without the need to develop theoretical equations (73).

An IPC method for the simultaneous separation of 20 typical antimicrobial agents was developed by an experimental design and models were built by the use of artificial neural-network to correlate the retention time of each analyte to the variables and their interactions. The neuronal models showed good predictive ability and were used, by a grid search algorithm, to optimize the chromatographic conditions for the separation (74).

Among chemometrical approaches, a multivariate-modified Simplex algorithm was applied for the optimization of the mobile phase used for the direct determination of bacitracin, a group of basic, cyclic polypeptide antibiotics in pharmaceutical formulations. The selected control variables were obviously the concentration of the organic modifier and the IPR in the eluent. The response variables were related to the peak area, the resolution, the asymmetry factor and the total number of chromatographic peaks. They were mathematically transformed so that they could be evaluated in an equivalent way. After successive trials, the algorithm stopped when optimum results were obtained and the prescribed mobile phase composition was then adopted (75).

Modelling of Retention as a Function of Analyte Nature

At variance with the modelling of k as a function of the mobile phase composition, the correlation and prediction of k as a function of the analyte nature has only very recently been initiated (76–78) and makes use of quantitative structure retention relationships (QSRR). QSRR is the method to identify the properties of solutes that control their retention. It allows the correlation k, measured at certain separation conditions, and solute descriptors such as physicochemical, topological, geometrical, and electronic factors, using multiple regression analysis. One kind of QSRR is based on the so-called linear solvation energy relationships (LSER). According to LSER log k is regressed against experimentally determined molecular descriptors. The first attempt to use LSER in IPC relied on a retention equation based on a mixture of stoichiometric and electrostatic models. A number of approximations were made (76–78). First, ion-pairing in the eluent was neglected, but this is at variance with clear qualitative and quantitative experimental results (50–58) and will be further discussed in the following. Similarly the presence of the IPR was assumed not to influence the retention of neutral analytes—they based this hypothesis on experimental results for neutral analytes, but these results were obtained for different ion-pair concentration in the mobile phase, hence they were not unbiased; it has to be taken into account that it was clearly demonstrated that this assumpion is only occasionally true (14, 17). Secondly, the only solute descriptor for ion-pair effect was the analyte charge, but it was demonstrated that the analyte charge status is not able to explain: (i) different theoretical curves when k is plotted as a function of the stationary phase concentration of the IPR, for different IPRs; (ii) dependence of the ratio of the retention of two different analytes on the IPR concentration; (iii) dependence of the k/ko ratio on the analyte nature if the experimental conditions are the same (12, 60); and (iv) the ion-pairing of peptides (58).

Furthermore the fitting coefficients of the solvation parameter model determined by the neutral solutes in the initial linear regression were used as fixed coefficients in the subsequent nonlinear least-squares fitting to obtain consistency, hence the trustworthiness and reliability of the coefficients, upon the inclusion of ionized solutes, was actually forced and not confirmed. The most compelling concern that arises upon analyzing the attempt made by Li et al. to use LSER in IPC is the fact that the ion-pair parameter estimates are not commented upon: since this fitting parameter has a clear physical meaning, its estimate is extremely important to judge the adequacy of the model. The authors took for granted that it must be zero for phosphoric acid because they say "no ion-pair effect should be observed"; they did not test this assumpion; a theoretical analysis of the meaning of this parameter (Eq. [9] of Ref (76)) confirms that it cannot be zero and actually phosphate ion was used as IPR (79) and it was demonstrated to give results similar to those obtained with trifluoroacetic acid (TFA) (57, 80–83). Moreover, the estimates obtained for different IPRs are not compared with the theotetical values of this parameter. If one takes also into account the fact that with enough processing and a sufficient number of fitting parameters nearly any conclusions can be derived from large quantities of data (84) it can be concluded that the use of QSRR in IPC is still in its infancy and much work has to be done yet to understand retention as a function of the solute structure. Noteworthy, based on their experience with LSER, skilled chromatographers very much doubted that the ability to quantitatively predict separation conditions to achieve an acceptable separation is an achievable goal even over the next decades, since change in the free energy of retention as small as a few hundredths of RT are chromatographically important (85).

FACTORS AFFECTING THE RETENTION BEHAVIOR

In the theory section we have rationalized the influence of the most important interlocking parameters that regulate retention; below we will consider the effect of stationary phase nature and eluent composition from a practical point of view.

Stationary Phase

Review of the literature highlights that the majority of separations are performed on conventional alkyl silica phases; along with the common C18 and C8 stationary phase, a C30 packing material was used for the analysis of inorganic ions (86) because the high hydrophobicity of the stationary phase provided adequate retention; a phenyl-hexyl phase was used to analyze heterocyclic aromatic amines (87). A C12 stationary phase with an embedded polar group successfully afforded the IPC baseline separation of seven tetracyclines (79). When C2, C8, and C18 stationary phases were compared, it was found that the maximum selectivity towards metal chelates was obtained using the C18 material (88). However, a C4 column was demonstrated to be highly eligible to reduce the chromatographic cycle time, including regeneration, for gradient elution of amino acids (89). Fluorinated polymeric adsorbents are emerging stationary phases. It has been reported that a fluorinated polymeric adsorbent was advantageously used for the purification of plasmid DNA (90). IPC was also performed on a classical ion exchange (IE) column to separate 17 anionic, neutral and cationic arsenic species in one chromatographic run, thanks to a multiplicity of retention modes on this packing material (91). Graphitized carbon stationary phases (PGC) were used in chiral IPC (92, 93) to enantioseparate amino alcohols, as outlined in Figure 4, but also to separate peptides and amino acids with perfluorinated IPRs (94–96) and inorganic anions in pharmaceutical compounds (97). It was reported that PGC exhibits unexpected and unique LC properties (94): this material is a very insoluble and stable LC support showing a highly ordered cristalline surface with delocalized electrons hence it is easily predicted to be more selective than ODS phases towards conjugate compounds; the retention pattern of a mixture of analytes may be completely different. At variance with classical RP packings it is possible to work under a wide pH range (1–13) and the equilibriation time for the adsorption of the IPR is faster (95). However, the stability of PGC used with fluorinated IPRs at low pH was considered questionable probably because fluorinated IPR oxidize it (98).

For the analysis of aqueous ions, a complexing agent, an alkyl-substituted macro-cyclic polyether, was loaded on a poly(styrene-divinyl/benzene) (SDVB) copolymer support (99); similarly, an additive that was at the same time an IPR and a complexing agent (di-(2-ethylhexyl) phosphoric acid) was loaded onto a RP column to separate the entire lanthanide series in a fission product mixture. A complexing reagent, α-hydroxy isobutyric acid, was used for gradient elution The stability of the stationary phase was tested and results were found to be reproducible (100).

The unusual normal phase was applied in chiral IPC (101–103), for the IPC of quaternary ammonium compounds (104), as well as for the determination of nitrite and nitrate (105). Similarly an aminopropyl, a cyanoethyl and a silica stationary phases were compared in the determination of alcohol denaturants; the cyanoethyl phase was selected and anionic IPRs were used to reduce rentention of cationic analytes suppressing their interactions with negatively charged silanols (106). Monoliths (single polymer pieces) feature significant advantages over particulate columns; due to their open channel structure they are more permeable and higher flow rates and moderate

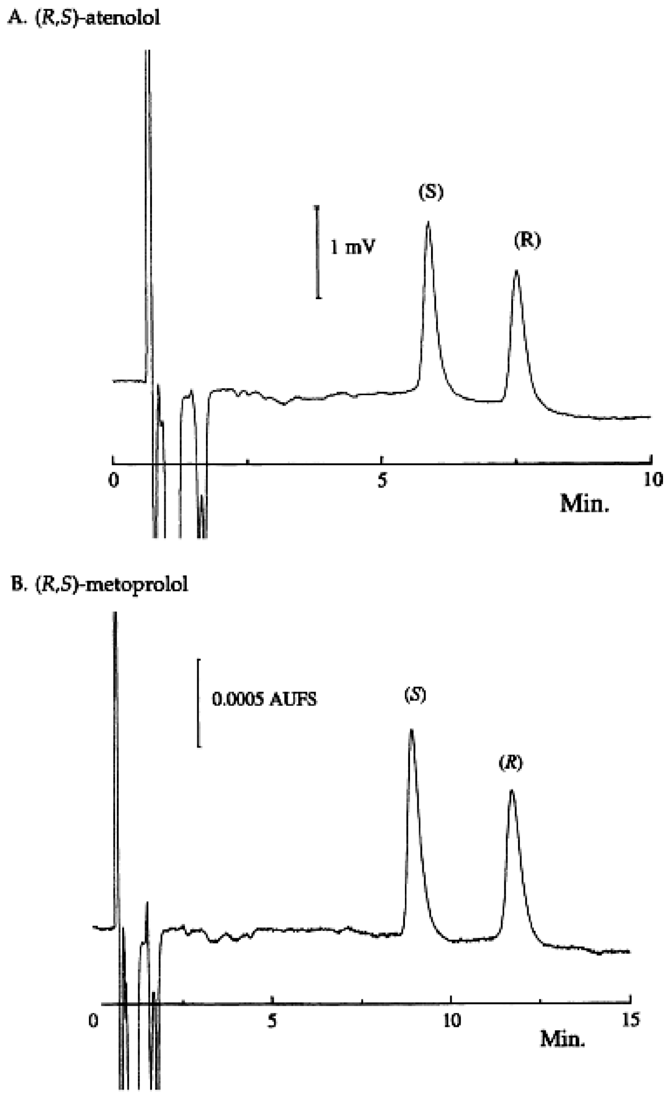

FIG. 4. Enantioseparation of atenolol and metoprolol. Solid phase: Hypercarb (5 mm, 10034.6 mm). Mobile phase: 5.0 mM Z-L-Asp–L-Pro and 9.0 mM NaOH in methanol. Flow-rate: 2.0 ml/min. Column temperature: 408°C. Reprinted from ref. (93) with permission from Elsevier.

backpressure can be used. A monolithic column was successfully used for the ultra fast (15 seconds) separation of common inorganic anions, with conductivity detector; the method was validated versus standard ion chromatography (107). A monolithic, poly(styrene-divinylbenzene)-based stationary phase was also used to separate peptides at high and low pH (108). A monolithic capillary poly(styrene-divinylbenzene)-based column was used in IPC for the direct molecular typing of genes (109) and for the analysis of ribonucleic acids (110). The chromatographic potential of a novel styrene stationary phase , monolithic poly(*p*-methylstyrene-co-1,2-bis(*p*-vinylphenyl)ethane) capillary columns for biopolymer separation via micro-IPC was assessed (111). Monolithic capillaries in IPC were demonstrated to bring increased sensitivity in the analysis of very low abundance of RNAs (112).

Perfusion stationary phases are constituted by highly crosslinked polystyrene–divinylbenzene, being capable to put up with backpressures up to 200 bar and to resist pH values ranging 1–14, high ionic strenghts and temperatures. Particles throughpores (600–800 Å) and diffusive pores (800–1500 Å) permit that the molecules cross the stationary phase particles by means of a combination of convective and diffusive transport making separations 10–100 times faster than in conventional chromatography but maintaining the resolution and column loading capacity. This stationary phase was profitably used to separate maize proteins (69).

Mobile Phase

Organic Modifier

The preferred mobile phase for IPC is a water-rich eluent; the purpose of adding organic modifier is to increase the analyte solubility, to enhance the wetting of the RP chromatographic packing, and to adjust analyte retention and selectivity. As explained in the theory section, by increasing the organic modifier eluent concentration a quantitatively predictable (21) retention decrease is obtained as widely witnessed by experimental results and depicted in Figure 1. Some representative experimental examples of this dependence can be found in refs. (86,113–118). Figure 5 clearly indicates that the retention decrease depends on the analyte nature. The reduction of the capacity factors with increasing organic modifier concentration in the eluent was stronger when acetonitrile was used compared to methanol, and this was ascribed to its lower polarity (114). Even if methanol or acetonitrile are the most used organic modifiers, tetrametilene oxide (119) has recently been tested in

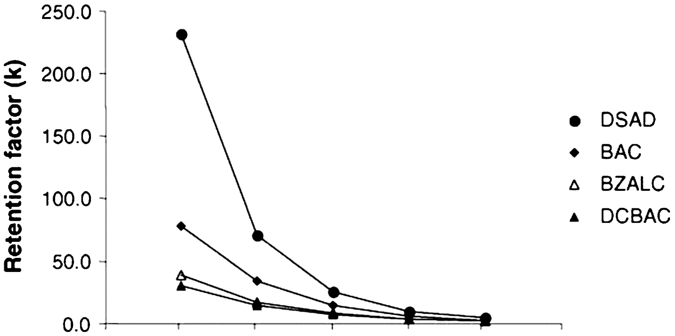

FIG. 5. Retention factor (k) vs. ACN concentration in an isocratic mode. Mobile phases: 25, 30, 35, 40, 55% v/v ACN-50mM phosphate buffer (pH 6.5) containing 2.5 mM THAHS. 2,4-DSAD is 2,4-disulfonic acid benzaldehyde di-sodium salt, BAC, BZALC, DCBAC are its impurities. THAHS is tetrahexylammonium hydrogen sulphate. Reprinted from ref. (113) with permission from Elsevier.

the IPC of sulfides and aromatic sulfonated compounds. This unusual organic modifier played an important role in adjusting retention.

Interestingly room temperature ionic liquids were used to control retention of adrenergic amines without using organic mobile phases and were proposed as environmentally friendly mobile phases (120).

Gradient Elution

Even if IPC has been traditionally performed in the isocratic elution mode, recent examples of gradient mobile phases prove that this strategy is valuable (68, 69, 76–78, 89, 94–96, 100, 113, 121–145) also because it avoids extreme pH conditions, lead a high peak capacity, and results in fast separations (146–149). Figure 6 outlines the analysis of the 20 underivatized amino acids in gradient elution on Hypercarb with nonafluoropentanoic acid as IPR. A step-gradient elution was also explored to change the eluent composition without a change in eluent concentration (99). In addition to the concentration gradient, a flow-rate gradient was introduced to shorten the cycle time of the chromatographic run, including the re-equilibration time needed because the organic modifier concentration gradient causes change in the distribution equilibrium of the IPR; this strategy was named "dual mode gradient IPC" (150). A different dual (concentration and pH) gradient allowed the sequential separation of all the lanthanides (151, 152). The gradient strategy also proved valuable for the positional isomer resolution (153, 154).

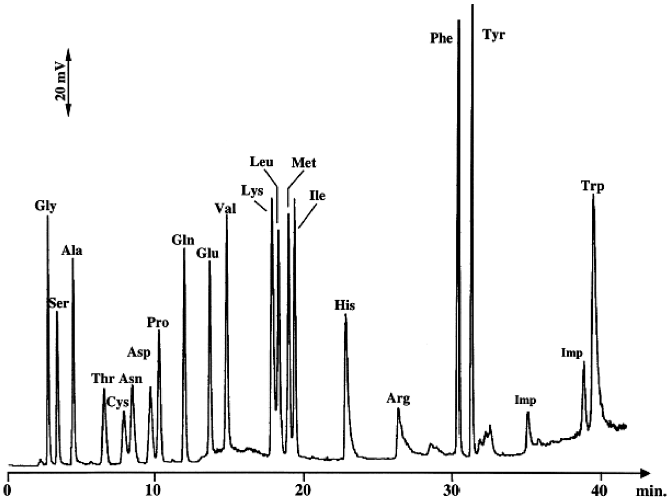

FIG. 6. LC–ELSD analysis of the 20 underivatized amino acids in gradient elution on Hypercarb (100 × 2.1 mm ID). Eluent A: nonafluoropentanoic acid (NFPA) 20 mM in an aqueous mobile phase. Eluent B: Acetonitrile. Gradient profile is: from 0 to 15% ACN in 10 minutes, then 26% ACN in 10 minutes and finally 50% ACN in a further 10 minutes. 50% ACN is maintained until the end. Column temperature is 10°C. Flow rate: 200 μL/min. Imp: Impurity. Reprinted from ref. (95) with permission from Elsevier.

A chemometric approach was followed to optimize the elution gradient program of the perfusion RP-HPLC method for the characterization of biogenic amines in wine (68) and of maize products (69).

Interestingly a gradient not only of the organic modifier concentration but also of the IPR concentration was crucial for the simultaneous quantitative analysis of several key classes of polar metabolites in metabolomics and for the determination of fluorescent whitening agents in environmental waters: a switch between IPC and RP-HPLC provided further selectivity (155, 156).

Different Additives

In order to mask heavy metal ions that impair the chromatographic performance of polar organic substances EDTA was successfully used (157, 158). EDTA was also used to suppress the masking of the nitrite peak by excess of iron (105), and it was also useful to prevent residual calcium ions from disrupt the ion-pair retention mechanism (159). Potassium tetrakis (1H-pyrazolyl)borate proved to be superior to EDTA for improving the peak shape of chelating analytes also because it could be better removed from the chromatographic column and because it coud be used in extreme pH conditions (160).

Unusual IPR

Conventionally, the majority of separations have been performed with standard quaternary amines of varying chain lengths as cationic IPRs for acidic analytes and alkyl or aryl sulfonates and sulfates as anionic IPRs for basic solutes. They used to rule "soap chromatography." Recently, atypical IPRs were tested: for example, it has been reported that for the analysis of pyridine-based vitamins in infant formulas dioctylsolfosuccinate gave a unique retention pattern (161) and a mixture of cationic and zwitterionic surfactant provided excellent separation of common anions on C18 (162). Ionic liquids were succesfully experienced as IPRs: both the cationic and the anionic components of the ionic liquid contribute to peak shape and resolution improving, but the ions oppositely charged to the analyte increase retention by ion-pair effects. It should be noted that when they are used as mobile phase additives at a millimolar concentration in the mobile phase, all specific properties of ionic liquids are gone and they become just dissociated salts. On adding these additives, water can become an environmentally friendly mobile phase and this kind of "green" IPC seems to be a promising development trend for chromatography (120, 163).

Among unusual IPRs two classes of compounds stand out among others: the homologous series of volatile perfluorinated carboxylic acids and chaotropic salts.

Perfluorinated carboxylic acids (76–78, 82, 83, 94–96, 121, 122, 125, 126, 130, 134, 136, 164–185) have been advantageously used and dominate peptide, amino acids, hydrophilic metabolites and ionogenic bases applications: their addition to the mobile phase affects the retention also changing the pH of the eluent (69, 108). Heptafluorobutyric acid in particular (125, 126, 134, 136, 175–178) was used for successful coupling to MS and to obtain additional selectivity compared to trifluoroacetic acid (TFA) or pentafluoropropanoic acid (PFPA). The volatility of perfluorinated IPR was also valuable when the evaporative light scattering detector was employed (75, 94, 95). Easy hyphenation with MS was also accomplished using volatile alkyl-, and dialkyl-, trialkylamines (108, 134, 141, 153–155, 186–198) often in the formate or acetate forms. Interestingly the volatility of TBA acetate was found sufficient for IPC-ESI -MS hyphenation (199).

Polarizable chaotropic ions on the Hoffmeister scale are interesting novel IPRs especially for the analysis of basic analytes (80, 82, 83, 200–205); they proved to be useful to achieve good resolution, selectivity and repeatability (206). Sulfonium and phosphonium IPRs showed single selectivity towards polarizable anions and their behavior was rationalized on the basis of their chaotropic or chosmotropic attitudes. IPC anion separation commonly relies upon ammonium-based IPR. Analytes across the Hoffmeister series from kosmotropic (iodate, chloride, nitrite) and intermediate (nitrate, bromide) to chaotropic anions (perchlorate, thiocyanate, iodide) allowed the characterization of sulfonium and phosphonium IPR: anion retention generally increases in the order tributylsulfonium < tetrabutylammonium < tetrabutylphosphonium. Their retention was found to be influenced by the kosmotropic/chaotropic character of both the IPR and the anion itself (200).

Quaternary phosphonium salts provided increased selectivity compared to ammonium analogs in the IPC of heavy-metal complexes of unithiol (115). The use of small lipophilic ions as IPRs has been found to be a promising trend for IPC: analyte retention is stronger as more chaotropic counterion, are used as IPRs. The effect of chaotropic ions on retention of oppositely charged analytes can be explained by: (i) the dynamic modification of the RP stationary consequent to their adsorption and (ii) the ion-pairing effect. The chaotripicity of an IPR, that is its ability to disrupt water structure, is related to its position in the Hoffmeister series. The Hoffmeister effect is stronger when the charge delocalization and polarizability are higher, hence we have the following sequence in order of increasing chaotripicity (206, 207): $H_2PO_4^- < HCOO^- < CH_3SO_3^- < Cl^- < NO_3^- < CF_3COO^- < BF_4^- < ClO_4^- < PF_6^-$. It follows that the retention of protonated amines increased, when chaotropic anions were used as IPRs, according to the above series (208) and the authors advocated for the dominance of the ion-pairing in solution because, even if chaotropic ions are not very hydrophobic, they can dehydrate more easily compared to kosmotropes. Since strong ion-pairing reactions require the exclusion of water molecules between the pairing charged species, it follows that chaotropic salts are able to give strong pairing interactions that allow increased interaction of the neutral complex with the hydrophobic stationary phase. The same view was also shared by Gritti and Guiochon (209–211) and by Flieger (206) and can explain the reason for which the perchlorate ion was a better IPR than dihydrogen phosphate for IPC of beta-blockers (80) and than trifluoroacetate for IPC of peptides (212). A comparison between these IPRs is detailed in Figure 7: it is clear that the absence of added salt or the presence of a salt such as NaCl, that is considered neutral in the Hoffmeister series, give a very different retention of probe analytes with varying net positive charge. It

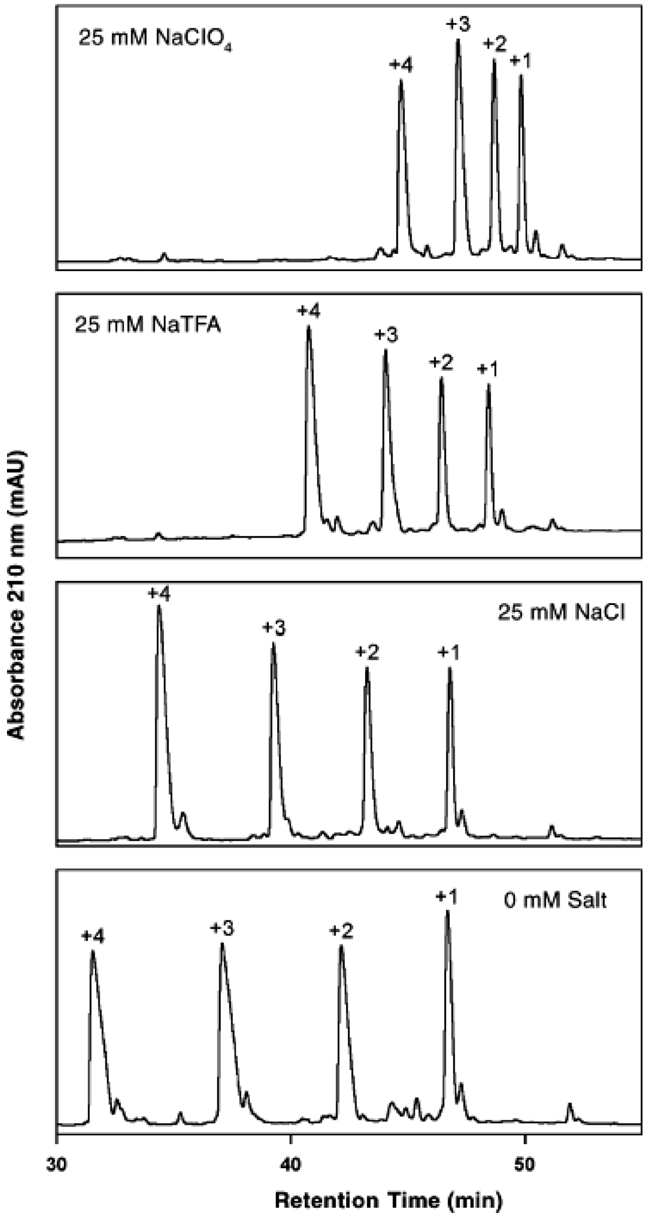

FIG. 7. Effect of salt on RP-HPLC elution profile of model peptides with varying net positive charge (+1, +2, +3, +4). Conditions: linear AB gradient (0.5% acetonitrile/min) at a flow rate of 0.3 mL/min, where Eluent A is 10 mM aqueous H_3PO_4 and Eluent B is 10 mM H_3PO_4 in 50% aqueous acetonitrile, both eluents containing 0 mM salt or 25 mM NaCl, NaTFA or $NaClO_4$; runs were carried out at 25°C. Reprinted from ref. (212) with permission from Elsevier.

was found that beside the type and concentration of the buffer counter-anion, the hydrophobicity of the basic analyte has an effective role determining to which extent the type and concentration of chaotropic anions will affect the retention of the protonated basic analytes: the lower the hydrophobicity of the analyte the lower the effect of the increase of chaotropic anion concentration (206, 213).

However, Kazakevich and co-workers demontrated the importance of the adsorption of the chaotropic ion onto the RP stationary phase (214) since the rank of an ion in the Hoffmeister series is also a measure of its tendency to accumulate at the stationary phase in RP-HPLC, that can be quantitated via its adsorption isotherm (207).

The effect of chaotropic mobile phase additives' nature on retention behavior of basic drugs (beta-blockers) was studied and it was found that they influence the selectivity and the efficiency (202, 215). When the chaotropic IPR was a ionic liquid at millimolar concentration the silanol screening by the cation and ion-pairing by the anion have beneficial influence on the chromatographic figures of merit; moreover, it is possible to play with beta-blockers retention by choosing different ionic liquid, which is a significant advantage not offered by classical silanol screening amines (216).

Interestingly also weakly chaotropic ions such as those coming from acetic (69, 132, 140, 201) or phosphoric acid were advantageously used as IPRs (57, 80–83) and the results were consistent with the theory of chaotropicity (202). Ammonium acetate proved to be useful in the analysis of sulfonated molecules present in samples coming from the photocatalytic degradation of methyl orange (217) and yielded satisfactory separations of sulphonic acids and anionic dyes (153, 218, 219).

Among bizarre IPRs we highlight the use of tris (hydroxymethyl)aminomethane in the determination of cyclamate in foods by ICP-ESI-MS: it was compared to different ion-pair reagents such as triethylamine and dibutylamine and it was chosen considering the sensitivity and time-saving (220). A divalent IPR, hexamethonium bromide was successfully used for the separation of sulphonates and carboxylates (116).

An unusual simultaneous usage of both anionic and cationic modifiers was reported to give more efficiency and shorten analysis time probably because the competition between the solute and the similarly charged IPR (221, 222).

Interestingly a dye, crystal violet, was used simultaneously as IPR and as the mobile phase component that allowed indirect photometric detection. This way common inorganic anions were analyzed and detected at the absorption maximum of the dye with classical RP columns. The affinity of the analytes decreased in the order $S_2O_3^- > SO_4^- > I^- > NO_3^- > Br^- > NO_2^- > Cl^-$, which is the same as that observed with anion exchange chromatography (71).

IPR Structure

Whereas IPRs of similar structure but with aliphatic chains of different length have similar effects, IPRs of different structure and charge have different effects on retention. At constant organic modifier and IPR concentration log k increases linearly with the number of carbon atoms in the chain of the alkyl sulfonate (4, 114). The shorter the IPR side chain is, the lower is its adsorption onto the stationary phase and hence the lower is its analyte retention, for a given mobile phase concentration of the IPR (12, 82, 83, 94, 95, 165, 223). This effect of IPR hydrophobicity on retention behavior of positively charged model peptides is illustrated in Figure 8. To attain a similar retention the chromatographer can use either higher concentration of a less hydrophobic IPR or a lower concentration of a more lipophilic IPR (10, 149, 168). Interestingly, a slightly different retention can be obtained for the same stationary phase concentration of the IPR and the same mobile phase, for different IPR chain length (9): this issue cannot be explained by electrostatic theories since the potential established at the surface is independent of the chain length. The extended thermodynamic approach quantitatively rationalizes this experimental evidence (12): a given stationary phase concentration of IPRs of different chain lengths corresponds to different IPR eluent concentrations, according to its specific adsorption isotherm. Since ion-pairing does occur also in the mobile phase this explains the different retentions for the same stationary phase concentration of different IPRs.

IPR Concentration

The shape of the plot k versus IPR concentration was a major challenge for model makers since the beginning. Knox and Hartwick demonstrated that it does not highlight the mechanism that governs solute retention (9). In order to clarify it the theoretical approach has to be followed as explained in the theory section.

The typical experimental plot of the capacity factor of an analyte oppositely charged to the IPR versus the IPR concentration shows a steep (quite rapid) increase, a maximum and a foldover and it was shown to be quantitatively explainable by the extended thermodynamic approach theory (12, 13). Obviously the maximum can also be missing, if the IPR concentration never reaches very high values. The theory also rationalizes (22) the experimental evidence that the greater is the analyte charge, the more marked is the effect of increasing IPR concentration on analyte retention, as expected on the basis of coulumbic interactions. Those interactions also explain the steep retention decrease of the capacity factor of an analyte similarly charged as the IPR with increasing IPR concentration. Some representative experimental results concerning the influence of the IPR concentration on analyte retention can be found in refs. (22, 80, 86, 94, 95, 99, 113, 114, 165, 168, 212, 224, 225). The extended thermodynamic approach theory was developed under the "trace conditions" approximation (12). If it is not fulfilled (analyte concentration too high, IPR concentration too low) experimental results may be at variance with the theoretical behavior even if such conditions are not usually chromatographically meaningful: for instance, the anomalous sigmoidal dependence of k

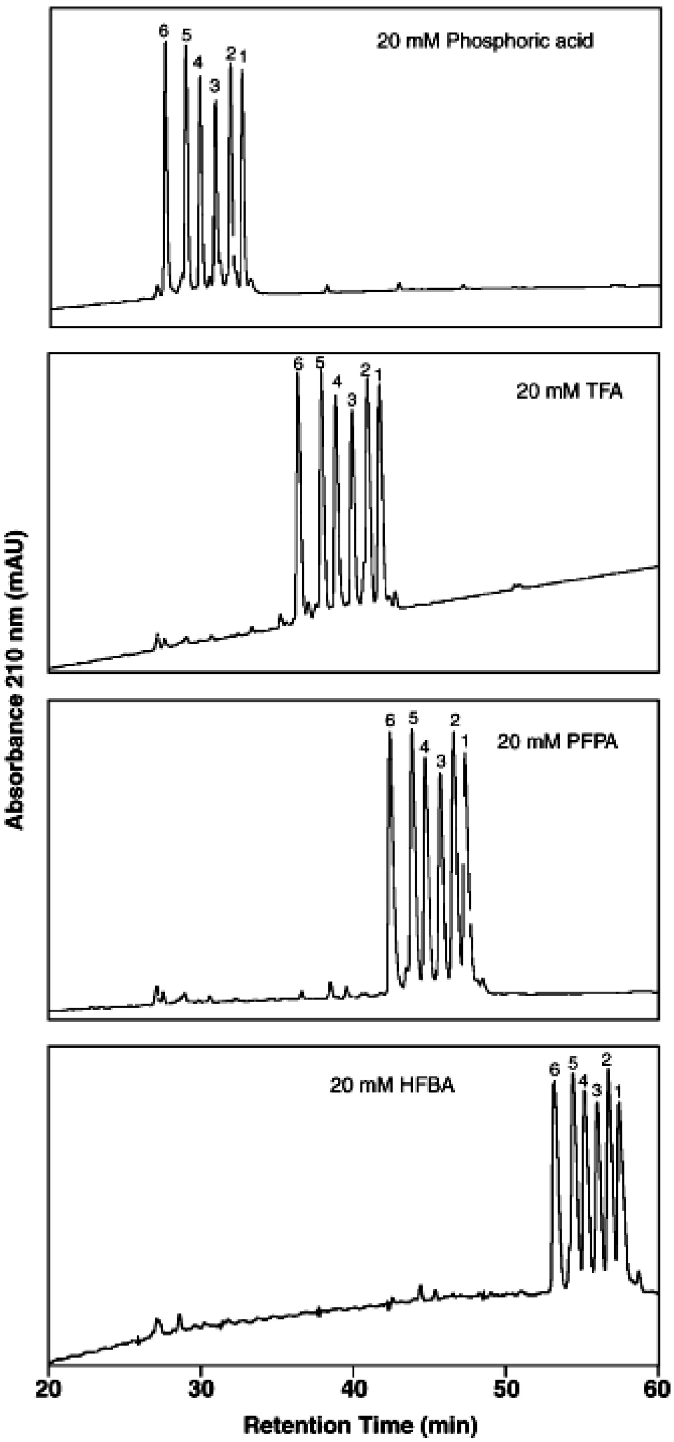

FIG. 8. Effect of ion-pairing reagent hydrophobicity on RP-HPLC behavior of positively charged model peptides. Conditions: linear AB gradient (0.5% acetonitrile/min) at a flow rate of 0.3 mL/min, where Eluent A is 20 mM aqueous H_3PO_4, TFA, PFPA or HFBA and Eluent B is 20 mM of the corresponding ion-pairing reagent in acetonitrile. The sequences of the peptides are shown in Table 1. Reprinted from ref. (165) with permission from Elsevier.

versus IPR concentration (3) at very low IPR concentrations is explained by the non negligibility of the analyte concentration compared to that of the IPR.

The gradual leveling off of the retention at high IPR concentrations was also explained for chaotropic IPRs as the result of a complete disruption of the basic analyte solvation; since this has been considered the driving force for increased retention the trend was easily predicted (82).

It follows from the theory that there is no optimum concentration for each individual IPR, rather it depends on all other system variables. For example, biogenic monoamines in rat brain striatum micro-dialysates were separated from interfering acids thanks to high concentration of the IPR; since it made the separation too long acetonitrile in the eluent was used to obtain reasonable separation times (226). For similar reasons the gradient strategy was often explored (see above) and the effect of tetraalkylammonium concentrations on retention of betacyanins in gradient IPC was found to be similar to that one expected for isocratic elution (149, 164) as it can be observed in Figure 9.

It was reported the positive influence of increasing IPR concentration on peak width and hence on resolution in peptides separation (168).

A switch between IPC and RP-HPLC that provided further selectivity was obtained by a simultaneous gradient of the organic modifier and of the IPR concentration (155, 156).

While it is a common practice to add a given concentration of IPR to the mobile phase, the possibility to add the IPR directly to the sample solution that will be injected into the chromatographic system was recently considered. This newly introduced strategy is cost effective because of the reduced chemical consumption and it was found to give satisfactory retention and to spare long column equilibration time. Moreover it was found particularly useful when IPC is hyphenated to MS, since the

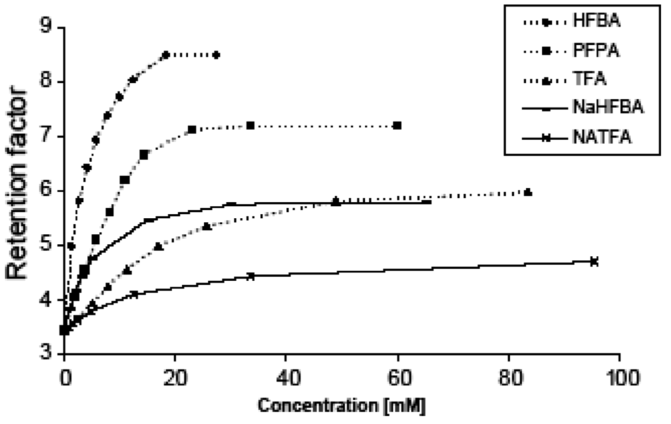

FIG. 9. Comparison of the retention factors of 17-decarboxy-betanin on the Luna C18(2) column in dependence on the concentration of perfluorinated carboxylic acids in the mobile phase containing 250 mM HCOOH and on the concentration of their salts at pH 1.7. Reprinted from ref. (164) with permission from Elsevier.

absence of IPR in the eluent minimizes the ionization suppression in the MS source, thereby increasing the sensitivity of the method (166, 227).

An fast ion chromatography (IC) of common anions was obtained by converting the reverse-phase columns into ion exchangers by coating the columns with hydrophobic surfactant typically every 3–5 days and eliminating the IPR from the eluent. An advantage of this approach is that the column anion exchange capacity can easily be varied by adjusting the acetonitrile content of the surfactant coating solution. Examples of this IPC mode can be found in ref. (228) and references therein. Permanent coating of the stationary phase has the additional advantage that it needs only a minimal quantity of the IPR, unlike the case of dynamic coating where the modifier solution is passed continuously; moreover, the eluted analytes are free from the ionic modifier. This strategy was found valuable in the gradient separation of entire lanthanide series in a fission product mixture on a reverse phase column coated with one of the best extractants available for lanthanides, di-(2-ethylhexyl) phosphoric acid (100).

It can be easily predicted that the trend of k versus IPR concentration in the unusual normal phase IPC is the opposite of the normal phase one (106).

pH

The acidity of the eluent is an important optimization parameter since it controls the ionization degrees of the solutes and hence the magnitude of electrostatic interactions and can be used to regulate selectivity.

If the pH changes in such a way that the analyte ionization increases, the RP retention sigmoidally decreases (82, 206), while the retention in the presence of a suitable IPR increases (149, 220, 229–231) because ion-pairing needs charged analytes to operate (80, 120). Unexpected pH dependence were ascribed to the presence of ionizable residual silanols on the surface of RP material (223).

Actually, with silica-based stationary phases the eluent pH is limited to a maximum of 7–8 due to the poor chemical stability of the chromatographic bed in alkaline solution since the nucleophilic attack of the Si-O bonds by hydroxide results in the erosion of the silica surface while the backpressure increases because of the formation of $Si(OH)_4$. Recently columns stable from pH 2–11.5 because of a bidentate linkage of the bonded C18 became available and allowed a fast (because of the high pH) IC of common anions, achieved by coating the columns with hydrophobic didodecyldimethylammonium bromide (228). PS-DVB monoliths pH stability is not an issue and both acidic and alkaline conditions can be used: this has a significant impact on selectivity in separation of peptide mixture (108).

It was found that at a pH so low that basic analytes are fully protonated, any further pH decrease by addition of $HClO_4$, CF_3COOH, or $H_3 PO_4$ led to a retention increase that involved a distortion of the classic sigmoidal plot, as outlined in Figure 10. Since the change of the counter anion concentration by simple salt addition produced the same effect on the analyte retention, although mobile phase pH remained constant, it was concluded that the chaotropic effect: (i) was not to be ascribed to the pH change but to the concentration of the chaotropic acidic modifier counter ion and (ii) depends on the nature of the anionic species but not on the source of the anion (acid or salt) (82, 224). However, when different perfluorinated acids and their salts were used as IPRs it was noticed, at variance with results in (82), that the acids are more effective in enhancing solute retention, compared to their sodium salts (164). Interestingly a pH gradient was tested to obtain the simultaneous detection of metal ions pre-complexed with a sulfonated azoligand (118), the quantitative analysis of several key classes of polar metabolites in metabolomics (155), and the baseline separation of six alkaloids (232). Along with the influence of the eluent pH on the analyte charge status, the influence of the mobile phase pH on the degree of ionization of the IPR also has also to be considered: ionorganic anions retention decreased above pH 5 because of the decrease of the ionized fraction of tetrabutylammonium hydroxide (86).

The pH was also very important in the IPC of complexes (233) because it controls the ionization of the ligand and, hence, the degree of complex formation and the original redox state of the metal (114, 115, 234, 235).

Temperature

Although column temperature is an optimization variable, it remains an underutilized separation parameter in LC, probably because results concerning the improvement of efficiency and selectivity have been controversial and the variation of the mobile phase composition provides an easy tool to adjust them. Moreover many silica-based columns are not thermally stable and this limits the chromatographically meaningful temperature range. A detailed theoretical analysis evaluated the effect of temperature on selectivity for several retention mechanisms in RP-HPLC and can be found in ref. (236). From the temperature dependence of retention in IPC, the variation of the thermodynamic parameters of enthalpy and entropy for the transfer process of the solute from the mobile to the stationary phase may be evaluated and a thermodynamic analysis of the process may be performed also via the van't Hoff equation

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \phi \qquad [21]$$

where ΔH and ΔS are, respectively, the enthalpy and entropy change for the retention interaction (113, 237). A typical van't Hoff plot is represented in Figure 11. In general, the variation of column temperature leads to a change in selectivity in IPC (238). For IPC of carboxylic acids a temperature increase results in a selectivity improvement only if the retention enthalpy is not dominant (236). For hydroxy-carboxylic acid hidrazides it was shown that a temperature increase has a strong effect on the separation and may lead to an inversion in the selectivity (239). Interestingly while the resolution of model peptides with +1 net charge improved with decreasing column temperature, the

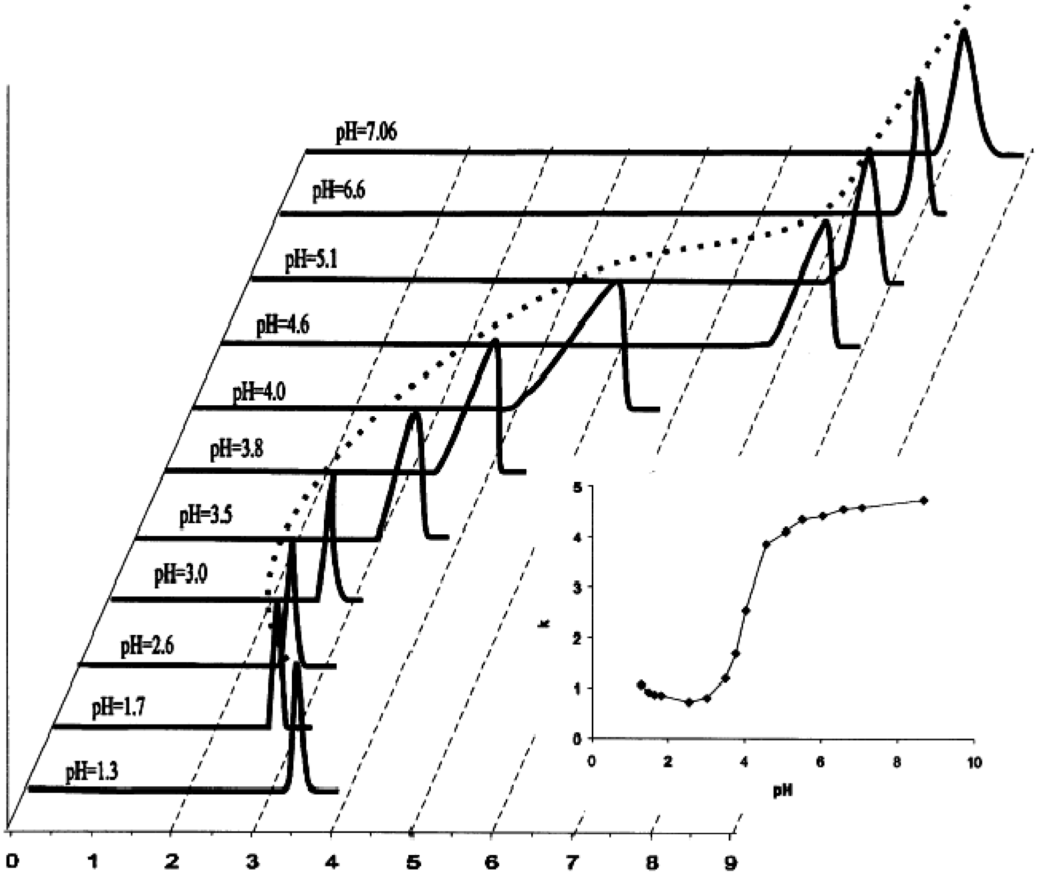

FIG. 10. Effect on retention of aniline when perchloric acid is used as the acidic modifier throughout pH region 1.3–7.1. Column 150 × 4.6 mm Zorbax XDB-C 18 mobile phase: acetonitrie—10 mM disodium hydrogenphosphate buffer adjusted with perchloric acid, pH 1.3–7.1 (10:90): flow-rate, 1.0 mL/min; 25°C; UV, 254 nm; sample: 1 μL injection. Reprinted from ref. (82) with permission from Elsevier.

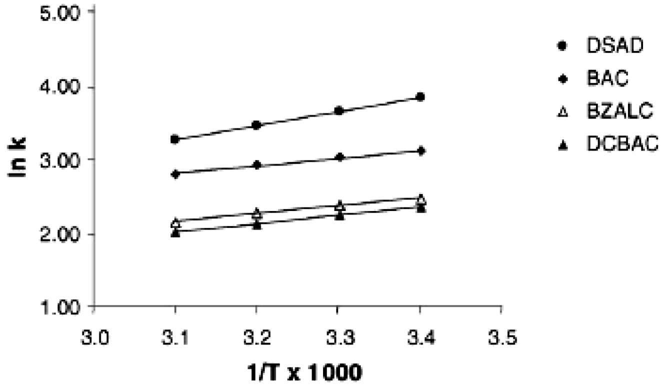

FIG. 11. Van't Hoff plot for 2,4-DSAD and some of the impurities in an isocratic mode. Mobile phase: 35/65 of ACN-50 mM phosphate buffer (pH 6.5) containing 2.5 mM THAHS. At 20, 30, 40 and 50°C. Symbols as in Figure 5. Reprinted from ref. (113) with permission from Elsevier.

opposite was observed for peptides with +3 net charge (170). Even if the selectivity remains unchanged, a high column temperature can be favorable for an increased efficiency (113) and a lower retention. In chiral IPC $T < 0°C$ was consistently used to improve enantioresolution (240). An increased resolution at 70°C was observed in IPC of peptides (57). A fast IPC of urinary thiocyanate takes advantage of a temperature of 45°C (241). For the IPC of underivatized carboxilic acids on a porous graphitic stationary phase the retention times decrease with increasing column temperature even if the analytes do not all exhibit the same behavior towards temperature; it follows that the separation is better at low temperature (95).

Interestingly the temperature was a crucial parameter to control DNA (109, 242–246) and RNA denaturation (139).

The column temperature was also an important parameter to obtain the best performance from the hyphenation of IPC with atomic absorption spectrometry in the on-line speciation of Cr(III) and Cr(VI) (138).

Elevated temperature enabled the application of higher flow rates due to the decreased solvent viscosity and thus column back pressure (111), but a resolution decrease was also observed (69, 247).

IPR Counterion

The volatility of the IPR counterion was demonstrated to be very important for IPC-ESI-MS hyphenation (see above), but it is also very important to regulate analyte retention since adsorbophilic IPR counterions reduce the net surface charge that develops as a consequence of the IPR adsorption; it follows that their presence reduces the surface potential. For the adsorption of an organic cation it was demonstrated that the essential factor that determines its retention is the number of negative charges on the pairing anion and secondly the hydrophobicity and hardness of the anion. The harder the anion (e.g., the smaller its size, the more poorly polarizable the anion with a limited charge delocalization), the lower the retention of the organic cation according to the following order that reflects well the global hydrophobicity of these anions: $HOOCC_6H_4COO^- \leq Cl^- \leq CH_3COO^- \leq HOOCC_2H_4COO^- \approx H_2PO4^-$ (210).

The synergistic contribution of both the cationic and anionic component of the IPR salt was evident when ionic liquids were used as IPRs (120, 163, 216).

Indifferent Electrolyte Concentration

Usually a buffered mobile phase is used to control analyte ionization via the eluent pH (see above). In Section 3.2.4 we described the potential of chaotropic salts to be used as novel IPRs. However, salts without a strong chaotropic or kosmotropic character (such as NaCl) that we may indicate as indifferent electrolytes do influence the analyte IPR retention as well. Increasing the salt concentration of the eluent results in (23):

(i) a decreased electrostatic surface potential, because the fixed surface charge is more shielded by the indifferent electrolyte ions in the electrical double layer: this causes a promotion of the analyte elution via a competition between salt ions and analyte ions for the adsorbed IPR.
(ii) a Donnan effect that helps the similarly charged adsorbed IPR ions to overcome the self-repulsion forces, hence at higher ionic strength the higher degree of IPR adsorption partially compensates the decrease in magnitude of the electrostatic surface potential.
(iii) salting-in or salting-out of the analytes because the addition of the salt results in a decreased or increased, respectively, surface tension of the eluent. IPC ions are usually salting-in agents since they decrease the surface tension of the eluent (3).

As a result of the interlocking issues above, IPC retention usually decreases with increasing ionic strength via the addition of an indifferent electrolyte as can be observed in Figure 2 (23, 113, 116, 117), according to the analyte charge (86, 116, 229). This effect can be observed in Figure 12: it underlines the fact that the log k' values of the positively chargerd melatonin and chlorthalidone do not depend significantly on the KH_2PO_4 concentration as both of the compounds do not interact with the similarly charged TBA. On the converse, log k' values of magnesium ascorbyl phosphate are almost linearly decreased with increasing phosphates concentration.

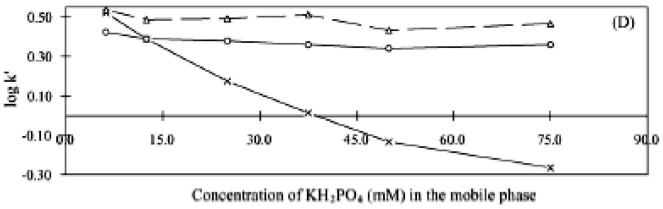

FIG. 12. Plots of the logarithm of capacity factor, log k', vs. concentration of KH_2PO_4 (in Mm). Magnesium ascorbyl phosphate (solid line, ×), melatonin (broken line, o) and chlorthalidone (broken line, Δ). Reprinted from ref. (229) with permission from Elsevier.

Even if added salt did not significantly affect analyte retention it was reported to be useful for improving the peak symmetry (116).

Table 1 summarizes the influence of the most important factors on analyte retention in IPC.

DETECTION

Derivatization, UV Visualization

Absorption in the UV and visible range is the standard detection mode, even if it is not universal, because many analytes lack suitable chromophores. UV visualization of non-UV absorbing compounds was often achieved via derivatization procedures: metal ions were detected via a post-column derivatization with 4-(2-pyridylazo)resorcinol (PAR), giving results in agreement with those obtained from atomic absorption spectroscopy (AAS) (248). An UV absorbing mobile phase (0.5 mM potassium hydrogen phthalate—0.015% triethanolamine, 3% methanol at pH

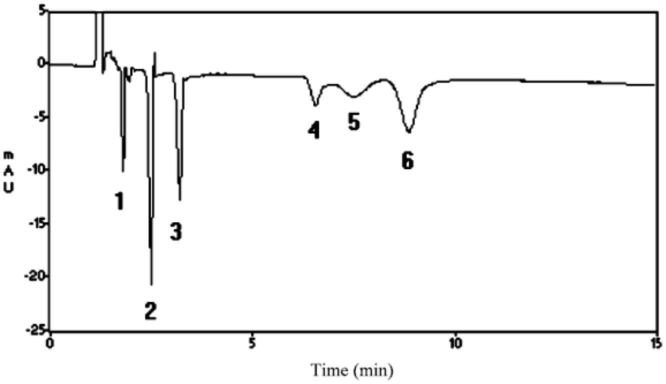

FIG. 13. Chromatogram of standard anions at 265 nm. Peak 1, hydroxymethanesulfonate (HMS) (148 μM); peak 2, chloride (506 μM); peak 3, nitrite (414 μM); peak 4, nitrate (203 μM); peak 5, sulfite (160 μM); peak 6, sulfate (252 μM). Sample volume, 10.0 μL. HMS is Reprinted from ref. (249) with permission from Elsevier.

TABLE 1
Factors that affect analyte retention in IPC

Factor	Effect on retention of an analyte in the opposite charge status to the IPR	Effect on retention of an analyte in the same charge status as the IPR
Absolute value of the charge of the analyte ion	Amplifies the effects of the parameters below	Amplifies the effects of the parameters below
Stationary phase	Increased lipophilicity or increased surface area increase retention	Increased lipophilicity or increased surface area increase retention
Increasing organic modifier concentration	Decreases more than in the absence of the IPR	Decreases less than in the absence of the IPR
Increasing IPR chain length/hydrophobicity	Increases	Decreases
Increasing IPR concentration	Increases up to a threshold value above which it decreases	Decreases
Changing pH	Increases when pH maximized ionization	Decreases when pH maximized ionization
Temperature	Retention decreases with increasing temperature, various effects on selectivity	Retention decreases with increasing temperature, various effects on selectivity
Increasing ionic strength with indifferent electrolytes	Decreases	Increases

7.9) allowed the indirect detection of sulphur and nitrogen anions in atmospheric liquids separated on a cetylpyridinium-coated C18 column (249), as illustrated in Figure 13. Crystal violet was used as IPR in the separation of common inorganic anions; indirect photometric detection at the absorption maximum of the dye allowed their detection down to 0.1 ppm with conventional HPLC equipment (71). Dithizone derivatives were used as chromogenic ligands for the quantitative determination of inorganic and organo-mercury compounds in aqueous matrices in under 12 minutes with an eluent consisting of 10 mM tetrabutylammonium bromide and 60:40 methanol water (250). The very sensitive indirect detection of polythionates and thiosulfate was based on their catalytic effects on the postcolumn azide-iodine reaction (251). Trace level bromate in bottled water was detected via a post-column flow reactor based on the oxidation of o-dianisidine (252); cysteine species in rat plasma were quantitatively determined by postcolumn ligand substitution, by measuring changing in the absorption at 500 nm (253). The use of a pre-column derivatization of sulfite with iron(III)-1,10-phenathroline complex was exploited in the direct photometric detection of sulfite in wines; the method was selective and sensitive (254). An easy and rapid pre-column derivatization method to convert the hydroxy-acids into their hydrazine derivatives was reported and validated (239). Biogenic amines derivatization was carried out using a post-column derivatization with 1,2-naphthoquinone-4-sulfonate (68). The post column reagent arsenazo (III), a metallochromic reagent, was used to obtain the photometric detection to monitor the eluting lanthanides and actinides (100, 151, 152).

Alternative Detectors

Even if conventional and diode array detector (DAD) UV spectrophotometers play the major role in the analyte detection, different kinds of detectors are receiving a grater share of credit.

Electrochemical detectors were used to analyze polar organic substances (157), iodide in urine (255), pramipexole in plasma and urine (256), morphine in postmortem blood (257), etimicin in commercial samples (247), and chatecolamines (258). For common inorganic anions (107), polimerase chain reaction products (259), aliphatic long chain quaternary ammonium compounds (260) and metallocyanide complexes of Fe(II), Ni(II) and Co(III) (261), detection was accomplished using conductivity detectors; Figure 14 illustrates the metallocyanide complexes chromatogram. Heterocyclic aromatic amines in soup cubes were determined with coulometric electrode array detectors consisting of four cells with different working potentials (87). For aliphatic amines potentiometric detection proved to give a sensitivity comparable with/to conventional UV (262). The amines potentiometric detection was successfully performed using macro-cycle-based liquid membrane electrodes (263). For heavy metals a novel amperometric detector based on the electrochemical transfer of the metal ions across an array of water/nitrobenzene micro-interfaces was advantageously used: the linearity was quite satisfactory, whereas the limit of detection was in the ppb range. More than eight metals are separated in less than 15 minutes on a C18 column using octyl sulfonate as IPR (264).

The evaporative light scattering detector is increasingly being used in LC as a quasi-universal detector eliminating the

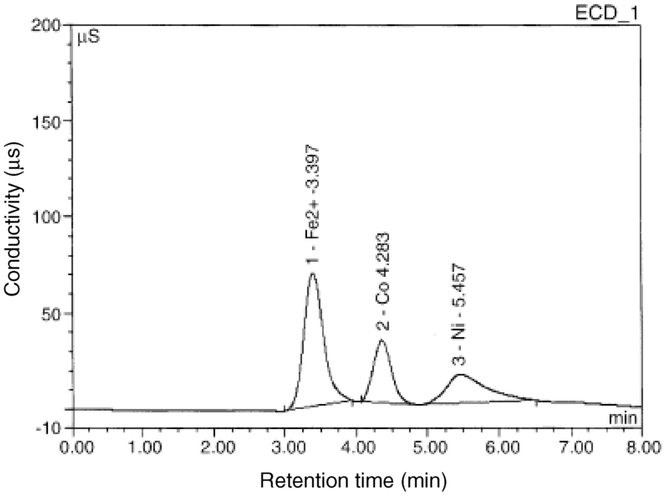

FIG. 14. Typical chromatogram obtained in the separation of cyano complexes of Fe(II), Ni(II) and Co(III). Mobile phase (2 mmol/L TBAOH, 1 mmol/L Na_2CO_3, 0.1 mol/L NaCN and ACN 77:23, v/v), flow rate of 0.7 mL/min, 0.2 mg CN^-/L for $Fe(CN)_6^{4-}$, 0.15 mgCN^-/L for $Co(CN)_6^{3-}$ and 0.1 mg CN^-/L for $Ni(CN)_4^{2-}$. Reprinted from ref. (261) with permission from Elsevier.

need for derivatization of non-UV absorbing analytes; since it demands the evaporation of the mobile phase prior to the light scattering step, volatile mobile phases are necessary, hence the method can be easily adapted to hyphenate IPC and MS. It was used to analyze small peptides (94) and underivatized amino acids (95**,** 130, 167) (see Figure 6), bacitracin in quality control of pharmaceuticals (75) as well as oligo-iota-carrageenans (265), unknown compounds in gentamicin (145) and bisphosphonates (266). It shows, compared to MS, lower investment and operating costs, easier operation and less maintenance. Sulfonate nathphtalene-formaldeyde condensates (267), benzene and naphthalene sulfonates (194), the disaccaride composition of heparin (268), metal-cyanide complexes (269), underivatized (270) and derivatized amino acids (127), quinine in drinks (271), tramadol in human plasma (272), 1′-hydroxybufuralol (205), albendazole marker residue in animal tissues (222), nucleic acids (273), riboflavin and the B6 vitamins (161) were all determined via a very sensitive and selective fluorescent detection. A post column derivatization fluorescence detection system was tested for the detection of amino acids, creatinine, bioactive amines, and nucleic acid bases (150). O-phthaldialdehyde was used for the fluorescence detection of fumonisins in corn (274), histidine (275), biogenic amines in cheese (276), glycyl-L-hystidyl-L-lysine, a growth factor, and its metabolite (277) biogenic amines and poliamines in vegetable products (135).

Biogenic monoamines in rat brain striatum micro-dialysates were separated from interfering acids with LOD ~ 200 pM thanks to the photoluminescence following electron-transfer detection technique (226) and the sensitive chemiluminescence detection proved to be very useful for the determination of dithiocarbamate fungicides by IPC (278).

HYPHENATION

Hyphenation of IPC to MS provides good sensitivity, structural information and unequivocal identification of analytes (95, 108, 110, 126, 166, 175, 179, 182, 186, 194-195, 267, 279–298).

For example, utilizing a volatile mobile phase, IPC-ESI-MS and simultaneous UV detection resulted in the high resolution separation of highly sulfated, heparin-derived oligosaccharides and provided sequence from a reading frame that begins at the non-reducing terminus of the heparin chain. High resolution MS afforded elemental composition of many known and previously unknown heparin-derived oligosaccharides (286).

The most common interface was ESI-MS (108, 134, 136, 139–141, 143, 145, 175, 176, 179, 182, 183, 186, 194, 197, 220, 265, 267, 280–283, 285, 288–298) because of the ionic nature of the analytes.

However, there are examples of APCI-MS applications (125, 185, 188, 192, 195, 279): quaternary ammonium herbicides were analyzed with heptafluorobutyric acid as IPR and both ESI e APCI-MS (125); moreover, APCI and ESI were demonstrated to yield very similar results when they were used in the determination of polyunsatured fatty acid monoepoxides (188). On the contrary, for the determination of mono- and disulphonated azo dyes, a comparison between various atmospheric pressure ionization interfaces demonstrated that ESI gave the best performance in terms of sensitivity reproducibility, although the structural information was poor (192). With negative APCI-MS and ESI-MS it was possible to unambiguously identify several acidic oxidation products of 2,4,6-trinitrotoluene (TNT) in ammunition wastewater and soil extracts (195).

Hyphenated IPC-MS methods have to be optimized not only with respect to the separation but also to the compatibility with on-line detection. The major concern of chromatographers who work in this field of research is the reduced signal that is involved in source pollution by non-volatile IPRs. Moreover, since the efficiency of droplet formation or dropletevaporation, which in turn affects the amount of charged ionsthat ultimately reaches the detector, can be impaired due to the formation of ion-pairs between the analyte and IPR (182), a severe ion suppression can prejudice the sensitivity of the method (181).

To overcome these problems some strategies were devised. Firstly, traditional IPRs were replaced with volatile IPRs. Even if non-volatile tetraalkylammonium salts generally provide the best chromatographic selectivity, volatile di- and trialkylammonium acetates or formats offered a good compromise between retention and selectivity on the one hand and the electrospray response on the other (108, 134, 141, 153–155, 186–197, 290), especially in capillary IPC-ESI-MS (198). Ammonium acetate was also used (153, 192, 217–219) and causes similar signal suppression of (poly)sulphonic acids as di- and trialkylammonium acetates but the separation selectivity is poorer (189). It was also demonstrated that the ion suppression effects are related to the instrument geometries and follow the order: Z-spray < orthogonal spray < linear spray. (189)

A different class of volatile IPR, perfluorinated anions, are being increasingly used in IPC-ESI-MS (95-96, 108, 125, 134, 136, 175, 179–181, 295).

Gustavsson et al. demonstrated that the chromatographic performance of TFA, heptafluorobutanoic and perfluoroheptanoic acids was comparable to that of conventional sufonate additives; moreover, the performance of the ESI interface was not impaired after 24 hours of continuous infusion. Nevertheless the ESI signal from most of the studied analytes decreased about 30–80% when these volatile IPRs were added to the mobile phase. HFBA produced better sensitivity than TFA but while the analyte retention increased with increasing the HFBA concentration, the ESI signal was found to be suppressed (182).

To minimize the signal suppression a post column addition of another volatile acid such as propionic acid was successfully tested: it gave more than six-fold enhancement of the ESI-MS response (183) and it was crucial for the quantitative determination of ceftiofur in milk (184). Kwon and Moini used atmospheric chemical ionization MS, which relies on gas-phase reactions rather than solution ionization, to overcome this problem when using NFPA as a mobile-phase additive (185).

Since volatile IPRs were reported to have some drawbacks concerning insufficient retention and separation (195, 279) other strategies to hyphenate IPC and MS were explored. To allow the chromatographers to exploit the favorable characteristics of TBA volatile counter ions such as acetate (199) or bromide (281) were used. For example, a micro-bore and capillary HPLC with very low flow rates (5–10 μL/min) with a conventional IPR (TBA bromide) proved to avoid source pollution (281).

The on-line removal of non-volatile ions by ion exchanger was also devised so that the process could be performed with favorable ionization conditions and without contamination of the interface (279, 282, 299).

Gangl et al. suggested the use of a nanosplitting device with high splitting ratios to reduce the signal suppression (300).

The possibility to add the IPR directly to the sample solution that will be injected into the chromatographic system without adding IPR in the eluent to easily hyphenated IPC-MS was recently considered: the absence of IPR in the eluent minimizes the ionization suppression in the MS source thereby increasing the sensitivity of the method (166, 227).

Figure 15 illustrates the results of selenium speciation, via ESI tandem mass spectrometry, in Brazil nut. It is clear that along with selected ion recording (SIR) the multiple reaction monitoring (MRM) is important because the transition of 198–102 is specific for a Se-molecule since a Se-fragment is lost (292).

In the analysis of nucleotide-activated sugars structural information was gained by both IPC-ESI-MS and IPC-NMR: the NMR spectrometer has to be equipped with a LC–NMR flow-probe cell; a stop-flow valve enables a precise parking of the LC peak of interest in the NMR flow-probe for the stop-flow experiments. The suppression of signals from ion-pair reagent was achieved by calculating automatically a selective pulse which enabled the suppression of all the signals from the solvent and triethylammonium acetate buffer (280).

IPC-ICP-MS was extensively explored for the speciation of phosphorous, arsenic, selenium, cadmium, mercury, and tin compounds (67, 91, 123, 171–173, 177, 178, 292, 293, 301–309), since it allows the element specific and sensitive detection. The same goal was reached in selenium speciation via an interface between IPC and atomic fluorescent spectrometry. This interface allows a thiourea reduction of all selenium species followed by conventional hydride generation scopus (137).

Unusual kinds of hyphenations were also reported. An at-line coupling of surface enhanced resonance Raman spectroscopy and IPC was used for the identification of basic dyes. The effluent, deposited on a TLC by means of a spray-jet interface, was analyzed by Raman spectroscopy and the presence of non-volatile IPRs resulted in higher intensities (310). Circular dicroism spectroscopy was a powerful hyphenated technique for selective detection of compounds possessing optically active adsorption bands and was used in the IPC of steroids (311). A two-dimensional electrophoresis and IPC allowed the identification of elements of the proliferating cell nuclear antigen multiprotein complex (312). A complete two-dimensional separation of acidic compounds in plasma was developed using column-switching with different separation modes in the first and in the second column. At the top of the latter a solute peak enrichment was obtained and IPC was performed (313). The speciation of arsenic compounds was obtained via IPC coupled with HGAAS (hydride generation atomic absorption spectrometry) (314). The hyphenation of IPC with atomic absorption spectrometry was also used in the on-line speciation of Cr(III) and Cr(VI) (138).

Flow injection (FI) was successfully coupled to IPC for batch complexation of metals with PAR: a PAR reagent solution is injected into a metal ion solution flowing stream. A portion of the PAR-metal mixture zone is then sampled with the HPLC injection valve for subsequent IPC and UV detection (315). If in this arrangement FI system was developed as the in-line precolumn for complexation, a similar hyphenated FI-IPC system was optimized to obtain, via flow injection sample processing, the on-line pre-concentration of trace metals on micro-column loaded with functionalized cellulose sorbent Cellex-P: since it exhibits fast kinetics of sorption processes it allows the rapid pre-concentration under flow conditions (248).

COMPARISON OF IPC WITH OTHER TECHNIQUES

IPC results usually compare to those obtained via different analytical strategies. IPC and capillary zone electrophoresis (CZE) were compared regarding the analysis of organic acids. Extreme stability, reproducibility and linearity are power points of IPC, while CZE showed better recovery; however, the statistical paired t-test demonstrated that for spiked urinary samples, there were no-significant differences for results obtained by the

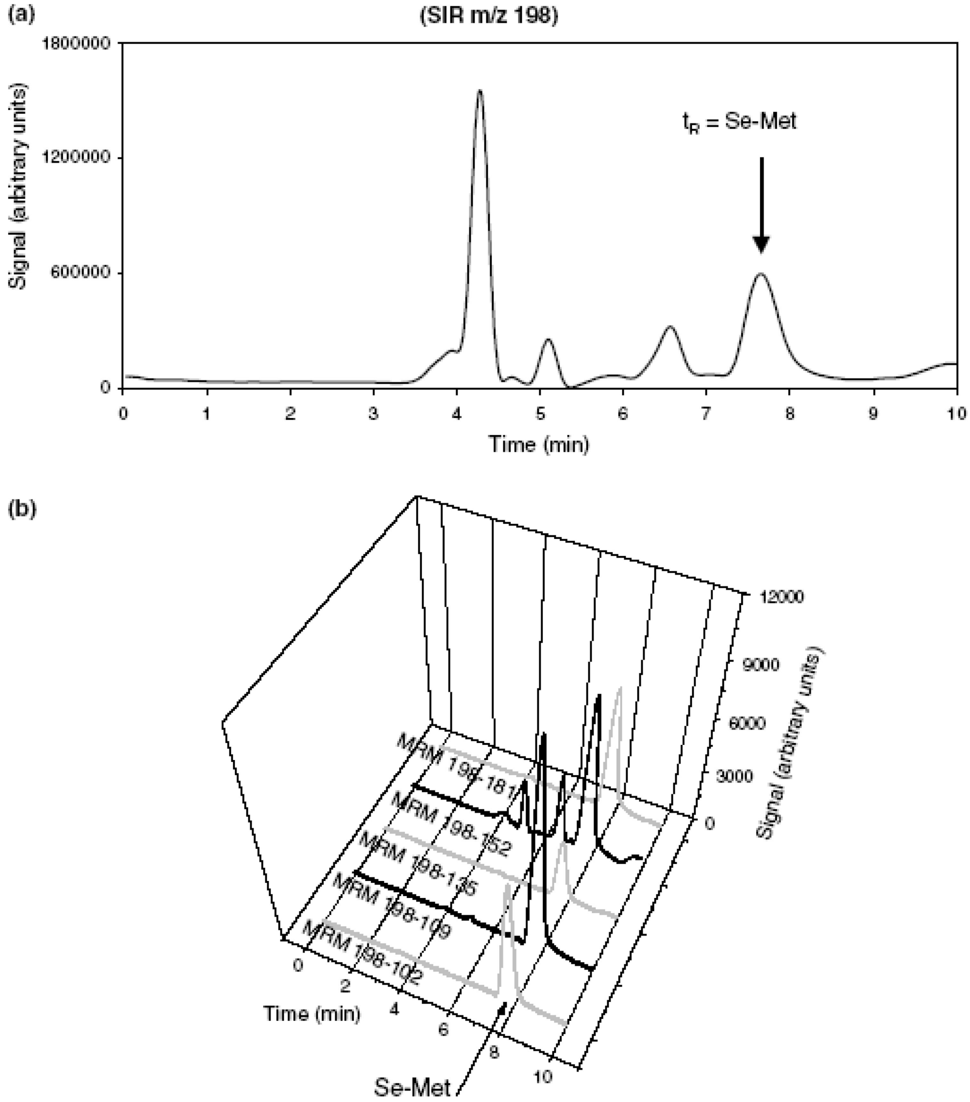

FIG. 15. (a) HPLC–ES-MS chromatogram of Brazil nut: SIR of m/z 198; (b) HPLC–ES-MS-MS: MRM of all product ions of Se-Met in Brazil nut. Reprinted from ref. (292) with permission from Elsevier.

two analytical strategies (316). Similarly, in the separation of stilbenes by IPC and CE, no significant bias were shown between them by t-test at 95% confidence level (317). IPC and CE were compared regarding the determination of EDTA and other complexing agents in cosmetic products. The HPLC method involves separation of their Fe (III) complexes by reversed-phase ion-pair chromatography on a C18 column using methanol–formate buffer (20 mM tetrabutylammonium hydrogen sulfate, 15 mM sodium formate adjusted to pH 4.0 with formic acid) (10:90, v/v) as mobile solvent. The CE separation was performed in a fused silica capillary of 50 μm ID with the total length of 50 cm with a buffer pH 5.5 at an applied voltage of −25 kV. In both cases UV detection was used. It was concluded that the concentrations found were comparable, but the IPC method displayed lower detection limits, while the advantages of the CE method were shorter analysis time and lower consumption of chemicals; the comparison between the figures of merit of both methods can be observed in Table 2 (318).

For the analysis of sulfonylurea herbicides, IPC proved to be superior to IC from the standpoint of separation performance (319). An ultra-fast IPC separation of common inorganic anions was validated versus standard IC (107). The same was done for the determination of nitrite and nitrate by normal phase IPC: the developed IPC method showed excellent sensitivity and had the advantage of avoiding the interference of chloride ions (105). The affinity of the analytes on a crystal violet coated RP column decreased in the order $S_2O_3^- > SO_4^- > I^- > NO_3^- > Br^- > NO_2^- > Cl^-$, which is the same as that observed with anion exchange chromatography (71). IPC and cation exchange chromatography of pyridinolines from bone hydrolisate showed a comparable

TABLE 2
Linear regression data of Fe(III)-EDTA, Fe(III)-IDS and Fe(III)-EDDS Reprinted from ref. (318) with permission from Elsevier

Complex	Range (μM)	Linear regression	Correlation coefficient (r)	LOD (μM)	LOQ (μM)
By HPLC					
Fe(III)-EDTA	0.3–2.0	$15.596x + 2575.5$	0.9974	0.1	0.25
Fe(III)-IDS	10.0–40.0	$3510.3x - 4464.9$	0.9906	5.0	10.0
Fe(III)-EDDS	5.0–20.0	$5327.3x + 17009$	0.9811	0.4	1.0
By CE					
Fe(III)-IDS	1000–3500	$1.0735x - 0.1835$	0.9982	300.0	900.0
Fe(III)-EDDS	200–700	$2.87x - 0.0103$	0.9961	50.0	150.0
Fe(III)-EDTA	200–700	$6.0219x + 0.0549$	0.9995	25.0	75.0

separation efficiencey, even if the drawback of IPC was a long equilibration time (320).

For the separation of aromatic carboxylic acids analytical data from IPC proved to be consistent with those obtained by conventional GC (142).

The performance of different analytical techniques for the separation and determination of haloacetic acids in a drinking water sample was compared; the advantage of IPC proved to be the fact that it did not require the pre-treatment of the sample to remove the interference of chloride ions (321).

In a quality evaluation strategy for multi-source active pharmaceutical ingredients starting materials, IPC was viewed as a stronger candidate among multiple analytical methods based upon the ease of operation (322).

When a hyphenated flow injection-IPC method was used to analyze Cr(VI), Co(II), Ni(II), and Cu(II) PAR complexes in chrome plating waste water, results were found in good agreement with that of AAS (315). A similar agreement is also reported in ref. (248). Akin results were obtained when IPC was compared to enzyme multiplied immunoassay technique for the determination of mycophenolic acid in plasma (323), and to amino acid analyzer results for histidine (275).

IPC and hydrophilic interaction chromatography were demonstrated to be complementary methods to analyze polar and non-polar glucosinolates from broccoli sprouts and seeds (324). However, for the anlysis of technetium species induced by spinach plants, IPC was not suitable compared to size exclusion chromatography because the strong interaction of the Tc species with the stationary phase under those experimental conditions (325).

Of the three chromatographic methods developed for analyzing cephamicins (ion-exchange, reversed-phase and ion-pair), the most selective are the last two even if all of them are fast, sensitive and accurate (326).

Compared to ion-suppression RP-HPLC, IPC separation of cyclic nucleotides showed a superior separation (281), but long equilibration times and poorer robustness were also observed in the separation of alkaloids (327).

The selective and sensitive analysis of sulfite in wine was performed via the pre-column derivatization of sulfite with iron(III)-1,10-phenathroline complex, IPC on a C18 column using acetonitrile-water (60:40) mobile phase containing 50 mmol/L $NaClO_4$ and 5 mmol/L acetate buffer (pH 5.0) and direct photometric detection. The IPC results agree with those obtained by the iodometric titration technique (254).

ION-PAIRING IN DIFFERENT (NON-EXTRACTIVE) ANALYTICAL TECHNIQUES

As regards supercritical fluid chromatography (SFC) with packed columns, several papers have been published in which the chromatographic retention mechanism is said to involve ion-pairing of amino alcohols, (328, 329), peptides (330), and sulfonates (331). Ethanesulfonic acid under SFC conditions was demonstrated to act as an ion-pairing agent with a crucial role in the enantioseparation of a wide variety of amines (332). Ion-pairing of sulfonates with ammonium salt additives was used in SFC also because it enhanced the solvating power of the mobile phase (333).

Ion-pairing was proposed to explain the sharpening of the peaks of eluted secondary amines and quaternary ammonium salts in SFC with polar bonded stationary phases (334).

Ion-pairing was also used to achieve on-line sample preconcentration in capillary zone electrophoresis: as the IPR moves through the sample zone, it effectively collects the oppositely charged analytes into a tight swept zone (335). Moreover, ion-pairing in CE can be useful to solve one of the major problems of this extremely efficient technique, that is the lack of versatility regarding manipulation of the separation selectivity. This issue was successfully studied for both organic (336–338) and inorganic (51) electrolytes. The CE enantioseparation of (a) (R,S)-Ala racemate and (b) (R,S)-Ala(P) enantiomers as N-DNP derivatives employing *tert* .-butylcarbamoylquinine as chiral IPR is illustrated in Figure 16.

Usually bi- or multi-dimension separations provide combined separation mechanisms that must be run individually. It was

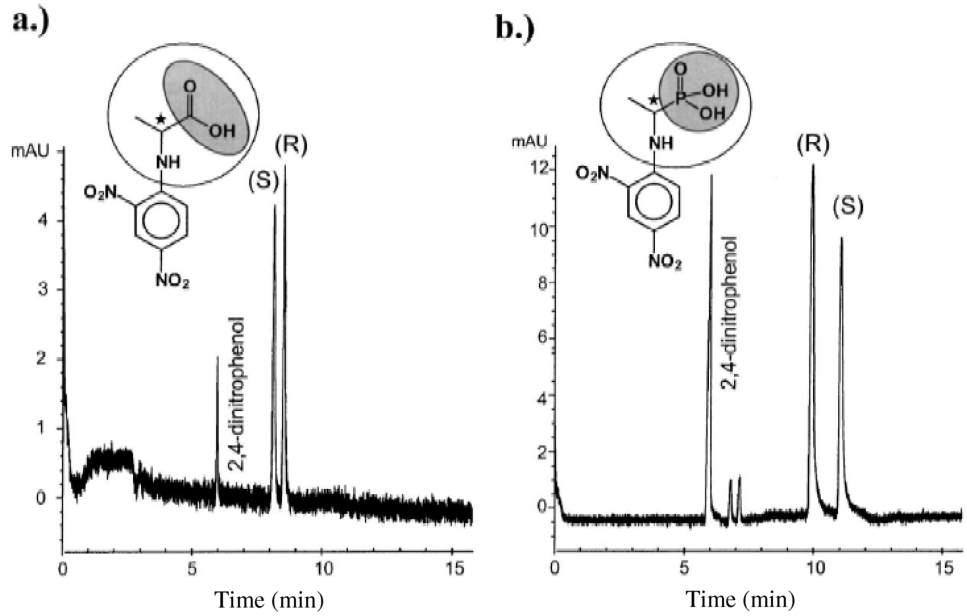

FIG. 16. Application of the conventional "total filling method" for the CE enantioseparation of (a) (R,S)-Ala racemate and (b) (R,S)-Ala(P) enantiomers as N-DNP derivatives employing *ter.*-butylcarbamoylquinine as chiral ion-pair SO under non-aqueous BGE conditions. Experimental conditions: 100 mM octanoic acid, 12.5 mM triethylamine and 10 mM *tert.*-butylcarbamoylquinine in EtOH–MeOH (60:40); T, voltage, 225 kV (23.5 μA); detection, UV 280 nm. Reprinted from ref. (337) with permission from Elsevier.

demonstrated that two compatible separation mechanisms, each based on a different analyte property (e.g. charge and hydrophobicity), that differ greatly in selectivity could be run simultaneously in the same single separation device and is equivalent to a bidimensional technique: peptide separation resulted from the combination of CZE and ion-pairing both located in the BGE. The optimization was accomplished easily in terms of counterion hydrophobicity and concentration. In comparison with other CE methods, this strategy represents a break from the chromatographic tradition of CE that provides the (chromatographic) hydrophobic interaction mechanism located at an interface (e.g., OT-CEC or MEKC). The major practical implication of this comceptually new separation strategy is the high peak capacity that can be obtained (57, 58, 169, 339).

NON-SEPARATIVE AIMS

Noteworthy, IPC was also used with non-separative aims. An unusual IPR (2-cyano-2-butylhexanoic acid) was selected to demonstrate via IPC that pharmaceutical efficiency of doxorubicin is related to ion-pairing at cellular level (340).

Denaturing HPLC (DHPLC) analysis is an ion-pair technique performed at a temperature sufficient to partially or completely denaturate DNA material. It has proved itself as one of the most reliable tools for DNA variation screening and allele discrimination, as well as quantitative measurement of gene expression and the analysis of single nucleotide extension products. Its sensitivity, accuracy and efficiency permit a fast, fully-automated and cost-effective detection of SNPs, insertions and deletions. It can be concluded that this newly introduced biothecnology compares favorably with conventional pre-screening methods and can be advantageously used for the comparative nucleic acid sequencing, evolutionary studies, forensics, and genetic studies. (109, 242–246, 294, 341, 342). An example of DHPLC is given in Figure 17.

IPC was also used to analyze the ligand content of benzamidine sepharose 4 fast flow media. Release of the ligand from the base matrix was obtained after hydrolysis for 180 minutes at 70°C in concentrated hydrochloric acid (343).

IPC demonstrates that the relative hydrophilicity/hydrophobicity of positively charged or hydrophobic peptide side-chains depend on characteristics of the peptides within which such side-chains are substituted (overall peptide hydrophobicity, number of positive charges) (83) and allowed the determination of the intrinsic hydrophilicity/hydrophobicity of amino acid side-chains in peptides in the absence of nearest-neighbor or conformational effects (344).

The IPR can also be advantageously used to determine, via its adsorption isotherms, the surface area and the monolayer capacity of a chromatographic packing, as well as the thermodynamic equilibrium constant for its adsorption. The method (41) champions previous attempts to quantitate the chromatographic stationary phase surface available for lipophilic ion adsorption because the non-approximated expression of the electrostatically modified Langmuir adsorption isotherm and the rigorous surface potential given by the non-approximated G-C theory equation were used. The theory also allows the estimates of the

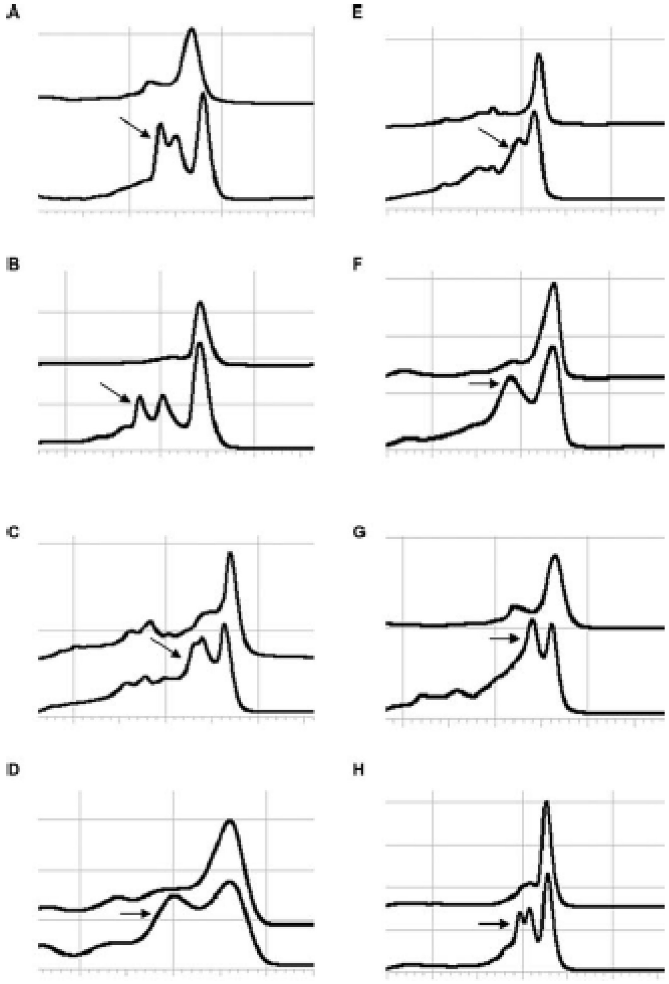

FIG. 17. DHPLC patterns of isolates to six drug-resistant genes. In each group, the first (upper) is wild-type (H37Rv), the second is a sample with mutation. Arrows (→) indicate patterns with a point mutation. A: *katG*A, kk11–2490 B: *katG*B, kk11-2492. C: *katG*C, kk11–2490 D: *rpoB*, kk11-2497. E: *embB*, kk11–2489 F: *gyrA*, kk11–2490. G: *pncA*, kk11–2488 H: *rpsL*, kk11-2489. Reprinted from ref. (246) with permission from Elsevier.

thermodynamic equilibrium constant for the adsorption of the amphiphile, the standard free energy of its adsorption, and the monolayer capacity of the packing.

IPC with a highly volatile IPR, pentadecafluorooctanoic acid, was also used in an on-line desalting-mass spectrometry system to trap via ion-pairing cationic hydrophilic analytes in a C30 trap column while unretained inorganic ions of the buffer pass through the column; finally the target compound was desorbed from the trap column in a blackflush mode and introduced into the MS (345).

A non-separative aim was also the determination of stability constants of metal chelates via IPC (346). Similarly, IPC proved to be a good alternative to the well established spectrophotometric, conductometric, and recently introduced CE methods of obtaining thermodynamic ion-pairing constants: the calculated ΔG° are reliable because they compare to non-chromatographic estimates of ΔG° for similar systems (24). However, IPC also played a foundamental role in elucidating the thermodynamics of the pairing process: the thermodynamic ion-pairing constants increase with increasing analyte chain length and decrease with increasing organic modifier concentration in the eluent; both results support a physical chemical description of the hydrophobic ion-pairing process at variance with the classical, Bjerrum-type modelling of the purely electrostatic interaction between inorganic ions (24). This evidence represents a break from the ion-pairing process tradition. It focused only on electrostatic interactions that were obviously dependent only on the dielectric constant of the medium and the size of the ions. The importance of the lipophilicity of the ions and the peculiarities of the hydrophobic ion pairing, due to the fact that the hydrogen-bonded water structure forces lipophilic ions together to minimize the structural perturbation, were also underlined in ref. (347) and references therein.

EXAMPLE OF APPLICATIONS

Inorganic and Organometalic Species

Many examples of applications of IPC have been reported during the last decade. It is instructive to observe the breadth of new applications concerning inorganic samples (67, 71, 86, 88, 91, 97, 99, 100, 105, 107, 114, 115, 117–119, 128, 137, 138, 151, 152, 162, 171–173, 177, 178, 197, 200, 219, 228, 233–235, 241, 248–252, 254, 255, 261, 264, 269, 291–293, 301–309, 314, 315, 318, 325, 346, 348–373).

The determination of various forms of the same element (speciation) is a challenging task for analitycal chemists. One of the most exploited procedures to improves the separation resolution is the complexation of metal ions. A better sensitivity can be obtained with conventional UV-VIS detection if absorbing ligands are selected (118, 233, 250, 315). Typically, complexation of metal ions is performed before injection but metal chelates have to be stable and kinetically inert (369). For examples, the formation of Fe(III) and Fe(II) chelates with pyridylazo and thiazolylazo reagents was exploited to speciate iron ions via IPC on a C18 stationary phase with sodium dodecanesulfonate as IPR (233). The simultaneous determination of Cr(III) and Cr(VI) $(Cr_2O_7^{2-})$ by IPC with octylammonium orthophosphate as IPR on a C18 separating column required the formation of the anionic complex between Cr(III) and EDTA prior to injection. (235). Potassium hydrogen phthalate, a more selective complexing agent for Cr(III), with a similar chromatographic system, allowed the chromium speciation in waste water (349). The resulting chromatogram obtained via UV detection can be observed in Figure 18. The hyphenation of IPC with atomic absorption spectrometry, with a solvent system comprising 15% v/v MeOH, 0.15 mM TBAA, 0.10 mM ammonium acetate, 2% ACN, and 4.1 mM H_3PO_4, gave Cr(III) and Cr(VI) LODs one order of magnitude

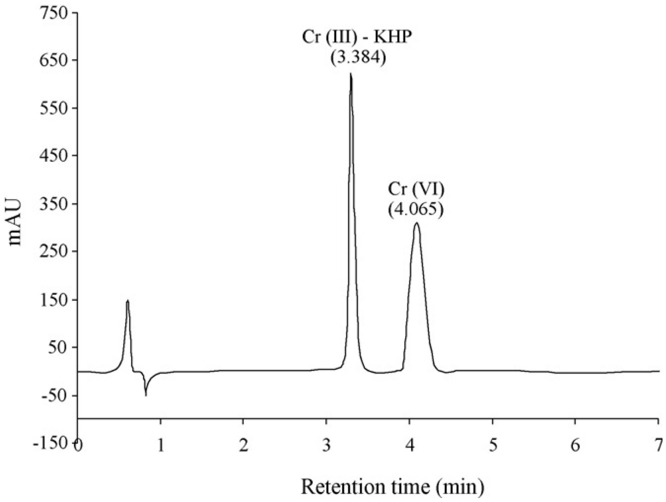

FIG. 18. Chromatogram of 5 mg/L Cr(III)–KHP and 10 mg/L Cr(VI). Chromatographic conditions: column, C-18 (Nucleosil-100, 3 μm, 60 mm × 4.6 mm); eluent, 5 mM octylammonium orthophosphate at pH 5.0 with 20% MeOH (v/v); flow rate, 1.0 mL/min; detection at 200 nm. Reprinted from ref. (349) with permission from Elsevier.

lower than those obtained via UV detection and good resolution in about 3 minutes (138).

Selenium speciation is fundamental to understand its biological role. The complexity of the matrices (garlic (137, 293), Brazil nut (292) (see Figure 15), mushroom (177), nettle (89), chives (171), B*rassica juncea* (178, 308), dill (172), yeast (372), human urine (302), and rye seedling biomass (305)) makes Se speciation particularly challenging: IPC was widely used for this purpose (67, 86, 137, 171–173, 177, 178, 292, 293, 302, 304, 305, 307, 308, 371–373). Common IPRs were ammonium salts (63, 137, 292, 293, 372) but perfluorinated carboxylic acids and particularly heptafluorobutanoic acid (HFBA) (171–173, 177, 178) proved to be more valuable for characterizing samples containing many different classes of organoselenium analytes (304).

Hyphenation with ICP-MS (67, 171–173, 177, 178, 292, 293, 302, 305, 307, 308, 371) has adequate sensitivity and selectivity but provides no structural identification. Hyphenation with ESI-MS-MS (372), with ESI-QTOF-MS (178), or with both ICP-MS and ESI-MS-MS (292, 304, 307) were explored. Hydride generation was used to couple IPC and atomic fluorescence spectrometry (137, 373). A chemometrical approach that used a central composite design allowed the determination of the optimum chromatographic conditions for the speciation of As and Se in tap water (67).

The speciation of As (67, 91, 301, 303, 314) via IPC hyphenated with ICP-MS (67, 91, 301, 303) or with hydride generation coupled with atomic absorption and atomic fluorescence was also widely investigated. A step gradient operation allowed to switch between heptanesulfonic acid as initial IPR and methanesulfonic acid. This allowed the complete separation of the arsenic compounds on a single run (303). Speciation of chromium (III) compounds (353), technetium species induced by spinach plants (325), and cadmium-bounds phytochelatins (306) were also faced with IPC.

Complexation was also used to obtain the chromatographic separation of different metal cations in the same sample or to obtain the stability constants of the complexes (346).

The separation of Fe(III), Cu, Pb, Zn, Ni, Co, and Mn via IPC was optimized. Complexation was not performed prior to injection but the ligand, tartaric acid, was included in the mobile phase. Retention decreased by increasing the concentration of the organic modifier or the ligand. In contrast, increasing the chain length of the alkanesulfonate used as IPR was found effective in enhancing the retention times The method was used for the determination of metals at the μg/L level in the analysis of natural water samples (114).

Among a variety of complexing reagents such as, for example, azo dyes, chelating ligands, diphenylcarbazone, unithiol, and azosulfonated ligand (115, 118, 197, 219, 234, 235, 348, 349, 367), cyanide is probably the most important. IPC separations of metallocyanides complexes of different metal ions in aqueous solution is now a customary technique (261, 269, 350, 351, 364) and can also be observed in Figure 14. The mobile phases usually contains cyanide, that prevent the dissociation of metallocyanide complexes (261, 350, 364, 368) and a suitable cationic IPR.

IPC has shortened the analysis time of lanthanides at trace levels, eluted as α-hydroxy isobutyric complexes, from days to minutes (100, 151, 152, 362).

Usually metal pre-complexation is time consuming and prone to contamination, and the operating costs are high. FI was successfully coupled to IPC for batch complexation of metals with PAR for subsequent IPC on a C18 column with tetrabutylammonium bromide as IPR. The method was used to analyze Cr(VI), Co(II), Ni(II), and Cu(II) in chrome plating waste water and reliable results in good agreement with that of AAS were obtained (315). A similar hyphenated FI-IPC system was optimized to on-line pre-concentrate trace metals in river water. The agreement with results obtained with electrothermal AAS determination was good (248). Pre-concentration of trace metals was also obtained using supported liquid membrane before IPC on a C18 column with octanesulfonate as IPR (352). Analytical approaches described above lowered the trace metal ions detection limits at sub μg/L (248, 315, 352).

Metal complex formation was also exploited for the determination of chelating analytes (318, 355).

Free metals were also amenable of IPC. More than eight metals are separated in less than 15 minutes on a C18 column using octyl sulfonate as IPR using a novel amperometric detector with limit of decision in the ppb range (264).

Since IC efficiencies continue to be lower than that of HPLC, instead of using an anion exchange column, RP materials under IPC conditions are the focus of interest of many investigations concerning the separation of inorganic ions.

Sulfur compounds were extensively analyzed by IPC (117, 119, 249, 251, 254, 356, 357, 365, 366).

Sulfur (IV) and sulfur (VI) species, such as sulfite and sulfate, play an important role in the atmospheric environment and in hot-spring waters.

Figure 13 illustrates the separation of sulfite, sulfate, hydroxymethanesulfonate (HMS), and other inorganic anions in atmospheric liquids. They were analyzed in less than 10 minutes with indirect photometric detection at 265 nm and with detection limits in the pmol range on a cetylpyridinium-coated C18, as it may be observed in (249).

Nitrite and nitrate were analyzed in spinach and lettuce by normal phase IPC with tetraethylammonium chloride as IPR (105). A fast ($<$50 seconds) IPC determination of nitrite and nitrate at trace concentrations in water samples made use of a 3.0 cm ODS column and a mobile phase comprising 20 mM tetrabuthylammonium chloride. The method allowed up to 60 analyses/hour which matches FIA analysis rate. Results are in agreement with those obtained via conventional ion chromatography (360). A similar system was optimized to separate up to eight UV absorbing anions with superb efficiencies (361). A fast ($<$1 minute) IC of common anions was obtained by converting a C18 column, stable from pH 2–11.5, into ion exchanger by coating it with didodecyldimethylammonium bromide and eliminating this IPR from the eluent. (228). Ultra-fast (15 seconds) separation of common anions was performed with a monolithic stationary phase using tetrabutylammonium-phthalate as IPC. This ultra-fast separation was successfully validated versus ion chromatography (107). Simultaneous determination of nitrite and nitrate in culture media of staphilicoccus strains (358) and in atmospheric liquids and lake waters (359) were also easily accomplished via IPC.

A chemometrical approach was used to collect sound retention data of common inorganic anions, separated on a RP column dynamically coated with an unusual IPR, that is a dye, crystal violet, that allowed the indirect photometric detection of the analytes at the absorption maximum of the dye. The affinity of the analytes was the same as that observed with anion exchange chromatography (71).

A very efficient column for simultaneous separation of common inorganic and organic ions was prepared by coating a RP column with a cationic IPR and incorporation of a non-ionic surfactant in the coating: this resulted in sharper peaks and a dramatic reduction of retention times, especially for organic anions (162).

Since the main drawback of ODS columns used under IPC to separate anions, compared to anion exchange stationary phases, is their incompatibility with eluents regularly used with conductivity detection, two strategies were devised to overcome this problem.

The potential of zwitterionic substances as eluents compatible with conductivity detection when anions are separated on RP columns with tetrabutylammonium as IPR, allowed a good separation of inorganic and and organic ions (162) The method was used to determine water-soluble anions in aerosol samples at concentrations as low as 0.3 mg/L (354).

Graphitized carbon packing, which shows excellent chemical and physical resistance, was used in the IPC of F^-, Cl^-, NO_2^-, Br^-, NO_3^-, SO_4^{--}, HPO_4^{--} and I^-. This way suppressed conductivity detection was possible. Calibration curves were linear with wide linear ranges. This method was used to accurately determine chloride, bromide and sulfate in pharmaceutical compounds (97).

Sulfonium and phosphonium IPRs showed single selectivity towards polarizable anions and their behavior was rationalized on the basis of the chaotropic or chosmotropic attitudes of both the anion and the IPR (200).

Interesting applications of IPC of common anions in real matrices were the determination of urinary thiocyanate and nitrate (241), the determination of iodide in urine with electrochemical detection (255), and the measurement of bromate in bottled water with post-column flow reactor detection (252).

Food Analysis

Anti-nutrients such as phytic acid and in its degradation products in nuts, cereals, legumes, etc. was obtained by IPC with gradient elution using tetrabutylammonium hydroxide as IPR and coupling to a double focusing ICP-sector field-MS (123); similarly, they were determined in rapeseeds and rapeseed meal by separating individual inositol phosphates (374) and the method was compared with two other methods.

Vitamins were determined in a variety of matrices; the analysis of water-soluble vitamins in infant milk was performed on a C18 column using octanesulfonic acid, with detection limits ranging from 0.02 to 0.10 μg/mL (375); dioctylsolsuccinate used as IPR facilitated unique retention of pyridine-based water soluble vitamins (161); the determination of total vitamin C in a wide range of fortified food products was peformed on a C18 column with decylamine as IPR and gave results in agreement with the official titrimetric method, with limit of detection at 0.1 mg/100 g and recoveries between 93 and 105% (376); folates from food were firstly purified by affinity chromatography and then separated via IPC (377).

A gradient IPC method to determine S-adenosyl-L-methionine in dietary supplement tablets was validated (124). Heterocyclic aromatic amines from soup cubes were separated on a phenyl-hexyl phase and detected with a coulometric electrode array (87). Biogenic amines in cheese were fluorimetrically detected thanks to a post-column derivatization with o-phthalaldeyde (276) while their separation that can be observed in Figure 3 was chemometrically optimized when they were determined in wines (68); a sensitive and selective method to determine 12 biogenic amines in a single 40-minute run, irrespective of the characteristics of the vegetal food matrix, was successfully validated. The range of linearity was up to 10 mg/L with correlation coefficient higher than 0.997 ($P < 0.001$) for all standard curves. The detection limits and the determination limit were below 0.07 and 0.2 mg/L, respectively, except for spermine. The precision of the method was satisfactory, according to Horwitz. Recovery was between 77 and 110% for all amines, irrespective of the product. A representative chromatogram under optimized conditions (10 mM octanesufonate mobile phase concentration with acetonitrile gradient elution, fluorimetric detection) can be observed in Figure 19 (135).

Glucosinolates from broccoli sprouts (324), fumonisins in corn products (274), food dyes (378), and carrageenans (265) are further examples of application of IPC in food analysis. A number of different compounds were analyzed in drinks, wines, juices and drinking water via IPC (116, 146, 239, 271, 379). Dermination of soybean proteins in commercial products was performed by a quite fast IPC, using a elution gradient and using a constant 0.3% (v/v) acetic acid as IPR. In order to apply the method for quality control purposes, a validation procedure was followed: since there is no certified reference material that can be suitably used as a standard for the determination of soybean proteins in commercial products a commercial soybean flour was used was used as standard (132, 133).

In the analysis and characerization of a commercial maize product a principal component analysis (PCA) did not enable a clustering of the samples studied. Thus, a classification of the samples was performed by a linear discriminant analysis (LDA). In the bid-imensional plot of the sample scores in the space defined by two discriminant functions, a separation of the maize samples into the four selected groups was achieved (69).

Furosine, an amadori compound related to heat treatment, was determined by IPC in yogurt and cheese samples (380).

An analytical method for determination of cyclamate in foods by IPC-ESI-MS has been developed and used to monitor the content of the artificial sweetener in foods. The method has high sensitivity, specificity and simplicity versus other methods reported (220).

The addition of tetrabutylammonium salts to the mobile phase can control the separation of complex mixtures of betacyanins and decarboxylated betacyanins, that are food colorants, in samples of food preparation or biological origin via gradient elution (149, 164).

Life Science

IPC proved to be a powerful tool in life science. Nucleotides (381, 382), cyclic nucleotides (281), picomolar quantities of nucleotides and nucleotide sugars extracted from mammalian cells (148), oligonucleotides (196, 223, 383), nucleobases (382), DNA fragments (384), RNA (110, 112), and RNA ocular metabolites (139) were all analyzed by IPC.

Polimerase chain reaction products were separated in less than 4 minutes by micro-IPC using triethylammonium acetate as IPR, and detected via a miniaturized conductivity detector (259). The purification of plasmid DNA was achieved with a fluorinated polymeric adsorbent capable of resolving supercoiled plasmid DNA from other plasmid isoforms, genomic DNA, RNA, proteins and endotoxins (90). DHPLC (see paragraph "Non-Separative Aims" above) is a relatively new IPC technique, which utilizes heteroduplex formation between wild-type and mutated DNA strands to identify point mutations. Heteroduplex molecules are separated from homoduplex molecules on a special column matrix with partial heat denaturation of the DNA strands. A denaturating mobile phase was used to obtain the fractionation of nucleic acid heteroduplexes and homoduplexes (243), to obtain an efficient genotyping (341, 342), to detect single base changes as efficiently as short deletions or insertions (244, 245), to analyze polymorphism (109, 291), and for point mutation detection in drug-resistant genes of mycobacterium tuberculosis (246), as illustrated in Figure 17. IPC was used in three mode of operations: (i) non-denaturing, (ii) partially denaturing, and (iii) fully denaturing to isolate and characterize five site-directed mutant amplicon; the method for DNA manipulations was versatile and facile and proved to be an adequate strategy for validating amplicons for mutation detection by denaturing HPLC (242).

Proteomics can also rely on IPC: IPC separation of peptides relies on the use of perfluorinated carboxylic acids, as can be observed in Figures 6–8, and in particular TFA with acetonitrile gradient at pH 2.0 (57, 58, 83, 94, 121, 165, 168–170). The perchlorate ion was demonstrated to be a better IPR than trifluoroacetate for IPC of peptides (212), as evident from Figure 7. Trypsic digest of various proteins (108), picomolar-order protein (385), amino acids and peptides (89, 95, 96, 122, 130, 167, 179, 180, 185, 253, 270, 275), and derivatized amino acids (127) were separated via IPC. Alkylsulfonic acids were more effective than the commonly used trifluoroacetic acid in the chiral resolution of 20 underivatized amino acids on an amylosic column (386). Monolithic columns based on poly-(styrene-divinylbenzene) (PS-DVB) were utilized both for preconcentration and analytical separation of peptides and proteins in column switching micro-scale HPLC. Trapping efficiency, especially for small and hydrophilic peptides, was optimized by using 0.10% heptafluorobutyric acid as solvent additive during sample loading. The applicability of the monolithic preconcentration column for multi-dimensional chromatography was tested by off-line two-dimensional separation, combining strong cation-exchange chromatography and ion-pair reversed-phase chromatography (387).

A "dual mode gradient IPC," using sodium dodecyl sulphate as IPR, was used to separate amino acids, creatinine, bioactive amines and nucleic acids bases: a flow rate gradient was introduced, along with the acetonitrile concentration gradient, to shorten the cycle time (150). Aliphatic long chain quaternary ammonium compounds that are active ingredients in lysing reagents for blood cell analyzers were analyzed by IPC coupled with suppressed conductivity and UV detection on a styrene–divinyl benzene chromatographic phase and the optimized chromatogram, obtained using 10 mM methanesulphonic acid as IPR and an acetonitrile gradient, can be observed in Figure 20 (260).

IPC proved to be a valuable aid in metabolomic, that is the most recent addition to the applied genomics and proteomics toolbox: since it involves the detection of all the metabolites in

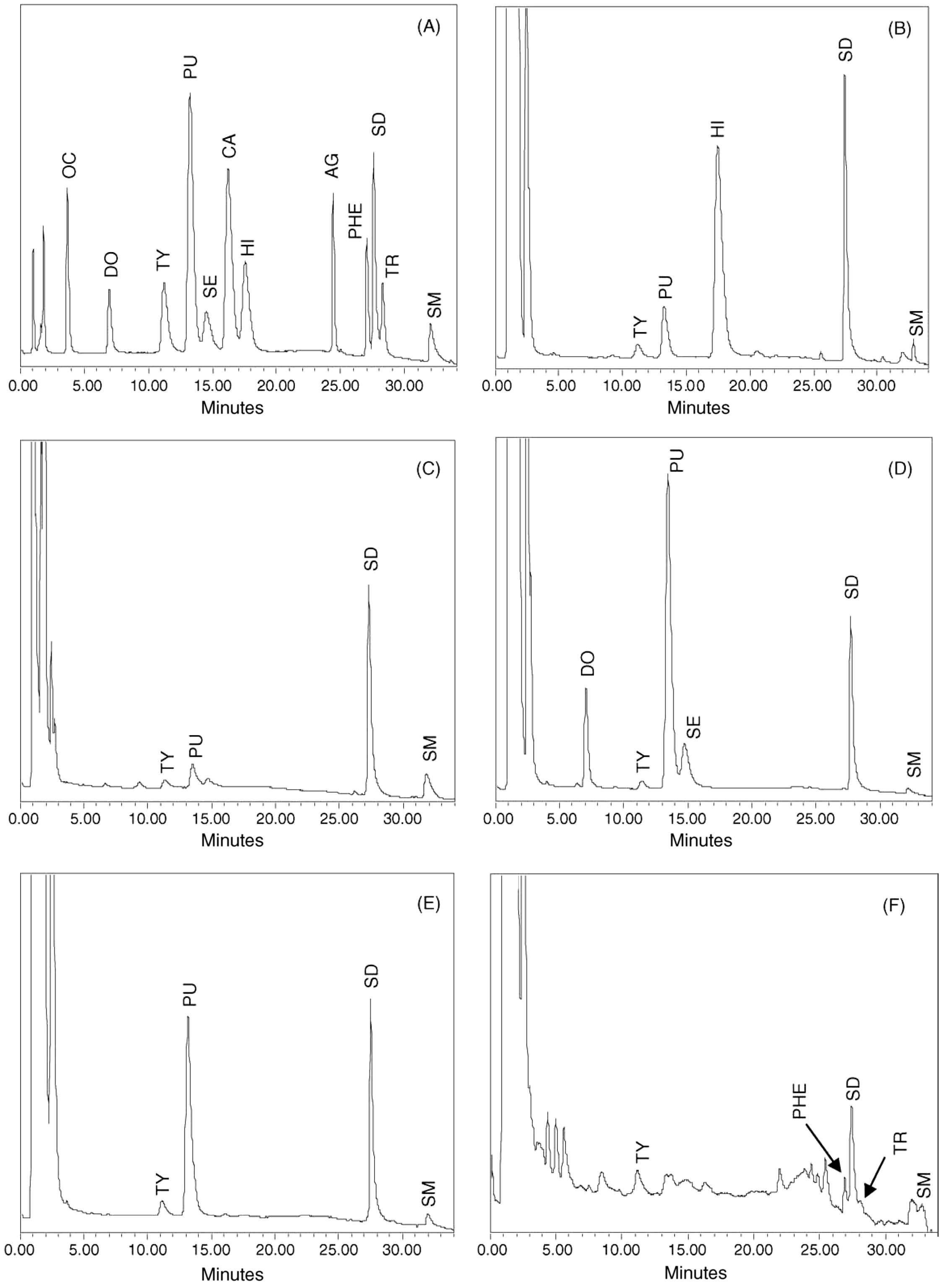

FIG. 19. Chromatograms of a biogenic amine and polyamine standard solution of 2 mg/L (A), and the spinach (B), hazelnut (C), banana (D), potato (E), milk chocolate (F) extracts. Reprinted from ref. (135) with permission from Elsevier.

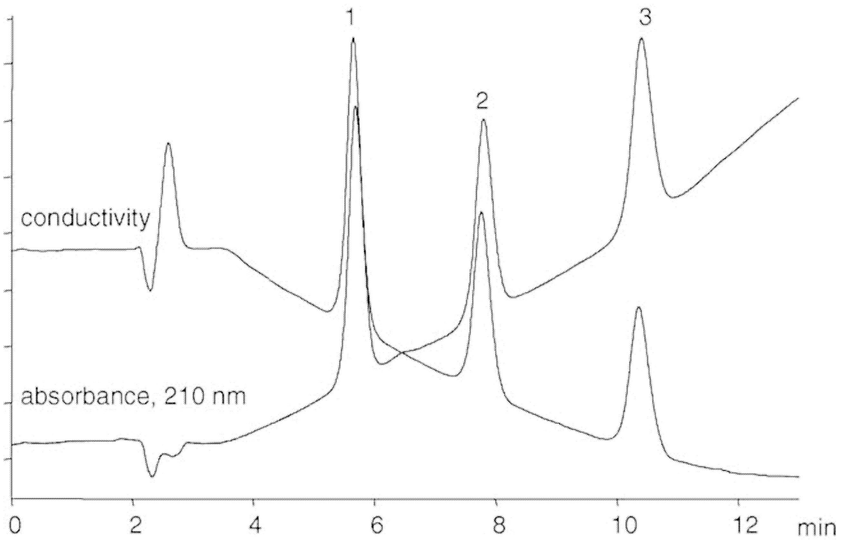

FIG. 20. Chromatogram for a standard solution containing approximately 100 μg/mL of the three alkyl trimethylammonium salts: (1) dodecyltrimethylammonium chloride (DTAC); (2) tetradecyltrimethylammonium bromide (TTAB); and (3) hexadecyltrimethylammonium chloride (HTAC). Reprinted from ref. (260) with permission from Elsevier.

a biological system a comprehensive analytical platform has to be developed to face this challenging problem. An IPC-ESI-MS method that allows the simultaneous analysis of an important class of metabolites in micro-organisms, such as nucleotides, coenzyme A esters, sugar nucleotides and sugar bisphophates was developed, optimized and validated; it made use of a gradient from 5 mM hexylamine in water (pH 6.3) to 90% methanol/10% 10 mM ammonium acetate (pH 8.5) (155).

Some guidelines for the analysis of genomic DNA by IPC-ESI-MS can be found in reference (287).

Pharmaceutical and Clinical Analysis

Amphetamines were widely investigated via IPC. A fully automated method using an on-line solid-phase extraction (SPE) and IPC with electrospray tandem mass spectrometry (LC/ESI-MS/MS) was developed for the analysis of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxyethylamphetamine, and 3,4-methylenedioxymethamphetamine in urine samples. Trifluoroacetic acid was added to the mobile phase as an ion-pairing reagent. Ion-pairing effectively minimizes the tailing effect caused by the interaction between the packing material and the protonated analytes. Good agreement between the results from the new method and a literature GC/MS method was obtained in the analysis of urine samples from drug users. The detection limit of each analyte, based on a signal-to-noise ratio of 3, ranged from 1–3 ng/mL (174, 298). The direct determination of p-hydroxymethamphetamine glucuronide in human urine was also studied (388).

The free form amphetamine was determined in rat brain with in-vivo micro-dialysis by IPC-ESI-MS. TFA was used in the mobile phase (acetonitrile–water, 10:90, v/v) as IPR. Since ion-pairing disguises the protonated amphetamine cations from the ESI-MS electric field as neutral molecules a post-column addition of propionic acid was found valuable to minimize TFA signal suppression effect on ESI-MS detection (183).

Room temperature ionic liquids were used to control retention of adrenergic amines, such as octopamine, synephrine and tyramine: they can be obtained from the citrus herbs and their extracts, and have been used in the treatment of obesity. The separation was carried out without using organic mobile phases and ionic liquids were proposed as environmentally friendly mobile phases (120).

The mechanism of catecholamine separation in IPC is relatively complicated. The variations in the mobile phase pH, concentration of counterion and organic modifiers can lead to extreme changes in the selectivity for the separation of ionic and ionizable biogenic amine compounds. These variation are, however, in agreement with the theory (258).

A fast and sensitive capillary liquid chromatography column-switching method with ESI–TOF–MS detection for the simultaneous determination of catecholamines such as dopamine, epinephrine, norepinephrine and serotonin was followed. The combination of the porous graphitic carbon material and the fluorinated strong acids which were required to get enough retention were demonstrated not be exploitable for easily oxidized compounds as the catecholamines (98). Free catecholamines determination in plasma samples was automated using dialysis and sample clean-up followed by IPC (389). Phenylephrine and related compounds were determined in human aqueous humor (390).

Biogenic monoamines in rat brain striatum micro-dialysates were separated from interfering acids with LOD ~ 200 pM, using photoluminescence following electron transfer (226).

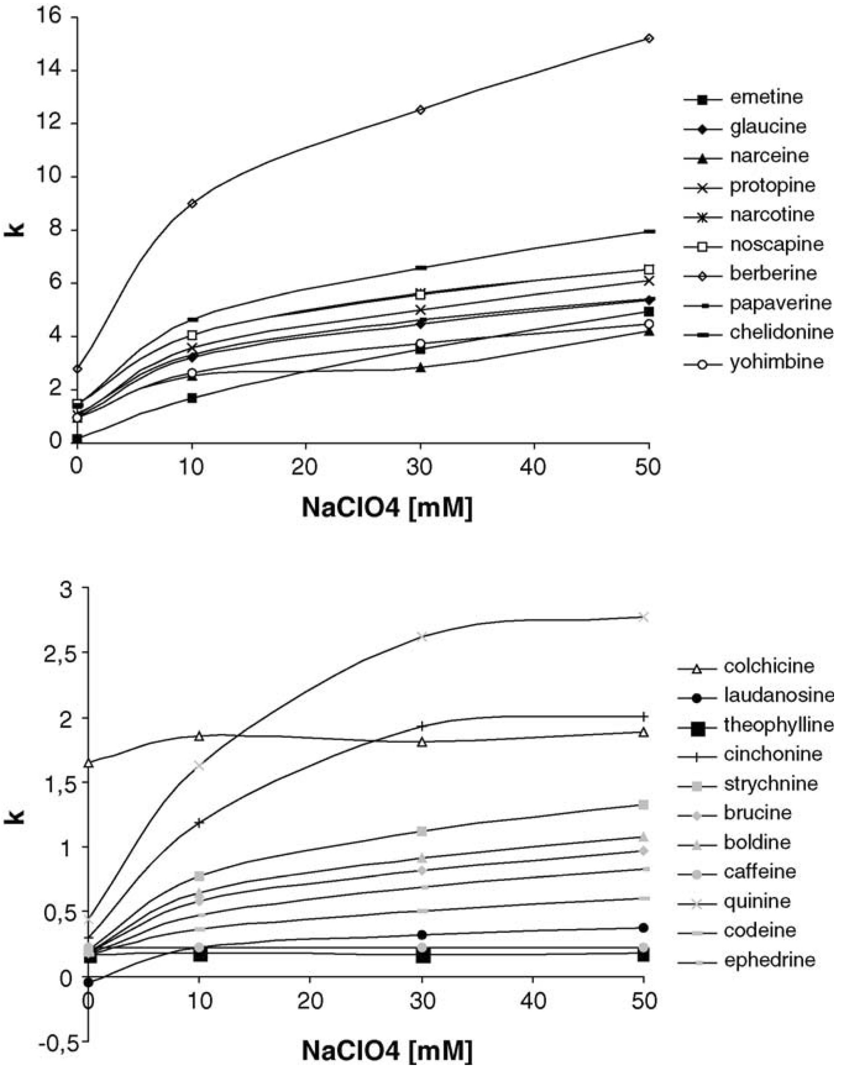

FIG. 21. Experimental retention factor for investigated alkaloids vs. perchlorate concentration in mobile phase: 30% ACN/10 mM phosphate buffer (pH 2.7). Reprinted from ref. (206) with permission from Elsevier.

The retention behavior of selected alkaloids from different classes was studied using chaotropic salt additives to the mobile phase with a C18 column. The addition of salts, such as hexafluorophosphate, perchlorate, and trifluoroacetate leads to the increase in retention, efficiency and separation selectivity of examined analytes. The influence of added salts is in agreement with their rank in the Hoffmeister series and the influence of the perchlorate concentration in the eluent on the alkaloids retention is detailed in Figure 21 (206). Interestingly a pH gradient was tested to obtain the baseline separation of six alkaloids from C. aurantium and E. sinica (among which are norephedrine, pseudoephedrine and ephedrine). The compounds are structurally closely related to catecholamines, thus they act as sympathomimetic stimulants; they are clinically used for the treatment of asthma, anaphylactic reactions or hypotension and can be found in dietary supplements (232). Solid phase extraction and subsequent IPC using lauryl sulphate as IPR and a electrochemical detector in series with a UV detector allowed a quick and reproducible quantitation of morphine and its metabolites from postmortem blood (257).

While it is a common practice to add a given concentration of IPR to the mobile phase, the possibility to add the IPR directly to the sample solution that will be injected into the chromatographic system was recently considered. This newly introduced strategy is cost effective because of the reduced chemical consumption and it was found to give satisfactory retention and to spare long column equilibration time. Moreover, it was found particularly useful when IPC is hyphenated to MS, since the absence of IPR in the eluent minimizes the ionization suppression in the MS source, thereby increasing the sensitivity of the method (166). An IPC-ESI-MS-MS method using volatile perfluorinated carboxylic acids as IPRs added directly to the sample solution and not incorporated into the mobile phase was developed for the quantitative assay of methadone in human plasma with a concentration range from 0.1 to 50 ng/mL. This strategy enhanced the efficiency of the separation and minimized the sensitivity loss due to the formation of ion-pairs because the IPR is not present in the eluent (166).

IPC was also valuable for the brain disease pharmacology: it was used to analyze picolinic acid and related compounds (158, 391), 46 neurotoxins, their precursors or derivatives involved in Parkinson's disease (147), pramipexole in plasma and urine (256), Huperzine-A (a biological potent, reversible acetylcholinesterase inhibitor for the treatment of Alzheimer's disease) (392), and nicergoline, clinically used for improving the brain metabolism (221).

Quaternary ammonium anti-cholinergics dugs were determined in whole blood and the matrix effect was taken into account (134).

Antibiotics were often investigated via IPC. Cephalosporins were determined by TLC-IPC (393). A rapid, specific and very sensitive liquid chromatographic assay has been developed to measure two cephalosporins: cefazolin or ceftriaxone in plasma. The ion-pairing reagent, cetyltrimethylammonium bromide, was used to prevent tailing of ceftriaxone (394).

An IPC assay for the analysis of ceftriaxone in cerebrospinal fluid of children with meningitis was developed and validated. The separation was achieved on a C18 column with a mobile phase (pH 7.4) comprising tetrabutylammonium bromide as the ion-pairing agent. The assay was linear from 0.5–50 μg/mL. The coefficients of variation for precision were $<4.61\%$. The accuracy ranged from 96.07 to 102.42%. The detection and quantitation limits were 0.019 and 0.065 μg/mL, respectively. (395). Three cephalosporins and two anti-TB antibiotics were isocratically separated on a C18 column using tetrabuthylammonium hydrogen sulphate (396). The quantitative determination of semi-synthetic cephamycins in human serum and urine by ion-exchange, reversed-phase and ion-pair chromatography were compared (326).

The analytical method for the determination of the aminoglycoside gentamicin in hospital wastewater via SPE and IPC-ESI-MS-MS relied on the use of the volatile HFBA as IPR (175). The structures of unknown compounds in gentamicin bulk samples were deduced with ion trap mass spectrometry by comparison of their fragmentation patterns with those of the available related substances and gentamicin reference substances; these compounds were separated via IPC using TFA as IPR (145).

The purity and content of etimicin, that is a water soluble aminoglycoside antibiotic, in commercial samples of etimicin sulphate, were determined via an IPC method using heptafluorobutyric acid as IPR; separation of eight components of etimicin was possible in less than 30 minutes; pulsed amperometric detection avoided derivatization (247). The ESI-MS-MS detection allowed the simultaneous IPC determination of neomycin and bacitracin in human or rabbit serum. Both substances and also kanamycin as internal standards elute at the same time but the hyphenation conveys specificity to the method; recoveries for a very simple sample preparation were high. The calibration range of these quite difficult detectable substances was 0.2–50 μg/mL of serum. The method was validated for both human or rabbit serum. At lower limits of quantitation inter-batch precision in human serum for neomycin was 12.05% and for bacitracin 11.91%, whereas accuracies were 99.9% for neomycin and 102.7% for bacitracin (397).

The IPC determination of fosomycin trometamol and its related substances in bulk drug was monitored by the ELSD (398).

Direct determination of bacitracin, that is a group of basic, cyclic polypeptide antibiotics in pharmaceutical formulations was chemometrically optimized using a multivariate-modified simplex algorithm. After successive trials, the algorithm stopped when optimum results were obtained and the prescribed mobile phase composition (H2O–ACN (73:27, v/v) containing 0.80 μL mL of TFA, at a flow rate of 1.0 mL/min) was then adopted; detection was performed via ELSD (75).

The IPC determination of norfloxacin in the presence of its decarboxylated degradation product and in mixture with tinidazole in bulk powder, laboratory prepared mixtures and in commercial samples was achieved on a C18 column with a mobile phase consisting of phosphate buffer pH 3.2 : methanol (3:1, v/v) containing 0.005 M sodium pentane sulfonate. (399).

A C12 stationary phase with embedded polar group successfully afforded the IPC baseline separation of seven tetracyclines using phosphate as IPR (79). Fast isocratic IPC of four tetracyclines and flumequine was obtained on a C18 column in 11 minutes. The peaks were narrow and without any tails although there were no chelating agents added to the mobile phase. Due to the chaotropic effect, the addition of potassium perchlorate allowed controlling selectivity (204). Anthracyclines were fluorometrically determined in plasma and urine using, without sample pre-treatment, using HFBA as IPR (400).

IPC was also used for the determination of adenine and pyridine nucleotides in glucose-limited chemostat cultures of penicillium simplicissimum (401).

Direct injection of wastewater sample into the IPC-ESI-MS system without an enrichment step to analyze acidic drugs and triclosan was possible because the volatile IPR tri-n-butylamine increased the sensitivity (297). The optimization of the IPC separation of 20 cosmetic preservatives was performed by experimental design and artificial neural networks (74).

Bisphosphonates, bone resorption inhibitors drugs, were the object of many investigations. The development of a chromatographic procedure for the analysis of bisphosphonates is not easy because most bisphosphonates are not chromophoric, hence UV detection is ruled out, moreover, it is hard to retain these charged solutes on RP and they are not amenable to GC analyses; bisphoshponates can chelate to metal surfaces, producing chromatographic peak tailing; different tetraalkylammonium salts commonly selected as ion-pairing agents in separation of bisphosphonates (159, 402–405) were replaced by volatile organic amines when ELSD was used (266, 406–408). Pyridinolines from decalcified bone were also amenable to IPC (320).

IPC was also used to analyze veterinary drugs in foods: for example, fenbendazole and its metabolites (409) and ceftiofur (184) were determined in milk, while albendazole marker residue was monitored in animal tissues (222).

The effect of chaotropic mobile phase additives' nature on retention behavior of basic drugs (beta-blockers) was studied and it was found that they influence the selectivity and the efficiency (80, 81, 202, 213, 215). When the chaotropic IPR was an ionic liquid the silanol screening by the cation and ion-pairing by the anion have beneficial influence on the chromatographic figures of merit; moreover, it is possible to play with beta-blockers' retention choosing different ionic liquid, which is a significant advantage not offered by classical silanol screening amines (216).

A two-dimensional electrophoresis and IPC allowed the identification of elements of the proliferating cell nuclear antigen multiprotein complex (312).

A method based on-line SPE capillary LC-ESI-MS has been developed for the determination of oxomemazine in human plasma (410).

Other interesting examples of the potential of IPC in the pharmaceutical and clinical analytical field are the methods developed to analyze: HIV protease inhibitors in human biological matrices (144, 411), HIV virus inibitor (412), 2′-deoxy-3′-thiacytidine (lamivudine) in human (413) and dog (414) plasma, 2′-fluoro-5-methyl-beta-l-arabinofuranosyl uracil triphosphate in human peripheral-blood mononuclear cells of hepatitis B virus-infected patients (296), cysteine species in rat plasma (253), ionic, highly polar and hydrophilic conjugated bile acids (187), steroids (311, 415), acidic compounds in plasma (313), organic acids as markers of abnormal metabolism (316), methotrexate (416), glycyl-L-hystidyl-L-lysine, a growth factor, and its metabolite (277), urinary thiocyanate (241), inhibitors of the nitric oxide synthesis (417), tegaserod maleate and its impurities in pharmaceutical formulations (418), ropivacaine metabolites (419) and other polar organic substances (101) in urine, tramadolin in human plasma (272), and tramadol glucuronides in urine (420), 4-nitrophenol and its glucuronide and sulphate conjugates in intestine perfusate (421) a complex mixture of analytes that range from uncharged to positively/negatively charged fexofenadine-D tablets (422), an alimentary opioid peptide was in cerebrospinal fluid (140), clonidine hydrochloride in hypotensor tablets of Zhenju Jiangya (423), frusemide and spironolactone in pharmaceutical preparations (424), sodium tanshinone IIA sulfonate, a cardiovascular drug in mouse plasma (425), the composition of heparin (268, 286, 299) and heparin sulphate oligosaccarides (198), long-chain fatty acyl-carnitines in tissue (176), monoepoxide derivatives of polyunsatured fatty acids (188), the pentose phosphate pathway metabolites (141), 2,4-disulfonic acid benzaldehyde di-sodium salt related impurities (113), niacine metabolites (426), L-carnitine in food supplement formulations (427), S-adenosyl-L-methionine in dietary supplemen tablets (124), ascorbic acid in gastric juice (428), folates in plant and animal tissue (129), magnesium ascorbyl phosphate and melatonin in cosmetic creams (229), and paralytic shellfish poisoning toxins in different matrices of novel food (429).

Environmental Analysis

IPC methods have been reported for the pesticide analysis in a wide spectrum of matrices: quaternary ammonium herbicides (quats compounds) (125, 126, 136, 181, 295) in water samples were detected using a quadrupole mass spectrometer with both ESI and APCI; detection limits complied with European Commission directives for water samples (100 ng/L) (125, 126). Quats were also analyzed in human whole blood with ESI-MS-MS to assess accidental paraquat poisoning (134), in human body fluids with ESI-MS-MS (295), and in olive oil with ESI-MS or UV detection (136); in all these cases a gradient elution with

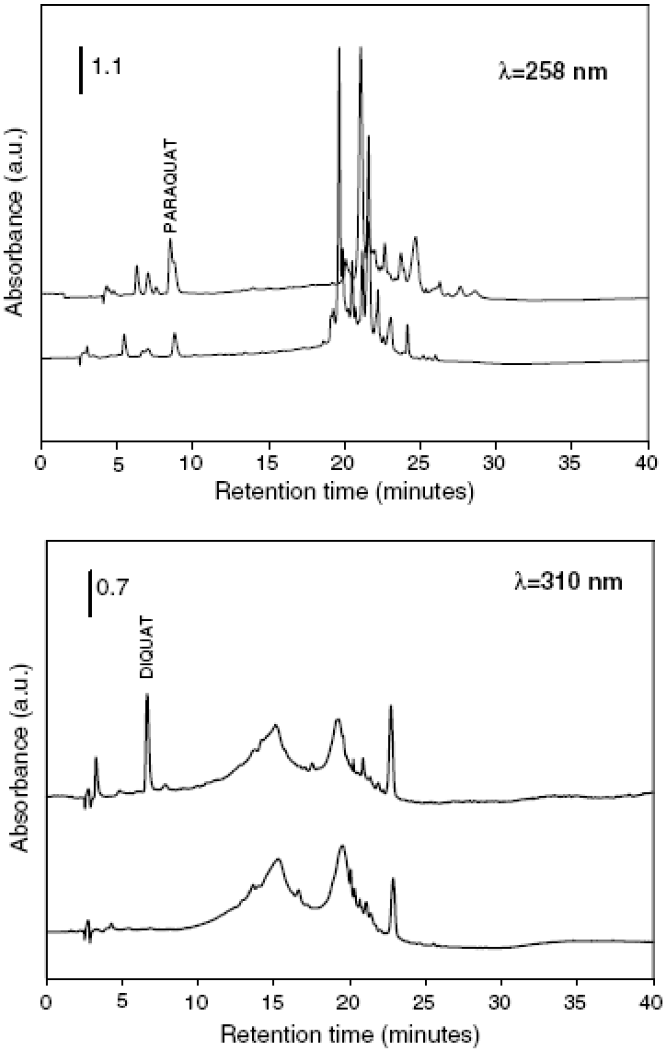

FIG. 22. Fortified sample containing paraquat (5 mg/kg, upper graph) and diquat (5 mg/kg, lower graph). For comparative reasons, the corresponding matrix before fortification is also included. Reprinted from ref. (136) with permission from Elsevier.

HFBA as IPR was performed. From Figure 22, that illustrates the chromatograms obtained for olive oil fortified samples, it is clear that the method is free from interference (136).

Phenoxyacetic herbicides and their main metabolites were determined in soil samples (230) and sulfonylurea herbicides in water (319). Dithiocarbamate fungicides in fruits and vegetables were analyzed using a conventional diode array detector using a derivatizing reaction with sulfite (430) or a sensitive chemiluminescence detection (278).

An IPC/tandem mass spectrometric method for the determination of ethephon residues in vegetables suggests to add the IPR directly to the sample solution to be injected into the chromatographic system: the absence of IPR in the eluent minimizes the ionization suppression in the MS source, thereby increasing the sensitivity of the method (227).

Monobutyl and dibutyl phosphate, which are degradation products of tributhylphosphate, were determined via IPC in tank wastes; tetrahexylammonium bromide was used as IPR and a pre-concentration procedure and results were confirmed via ion chromatography with conductivity detection (431). Ion-pair extraction and ion-pair chromatography were combined for the determination of phosphoric acid mono- and di-esters in municipal wastewater. It was evident that matrix effects during ESI may alter the signal intensity of a given analyte in the analysis of real samples. In order to find out these effects the response factors obtained from standard addition of the analytes into a wastewater extract (R2) and into pure water (R1) were compared. Since the relative responses (R2/R1) of the analytes were significantly decreased, their reliable quantification from aqueous environmental samples was performed with standard addition; it was found that even tertiary treatment does not ensure complete removal of the studied compounds that may probably originate from the microbial hydrolysis of phosphoric acid trimethylesters used as flame retardants (143).

Organophosphorus chemical warfare degradation products were amenable to hyphenated IPC and ICP-MS equipped with a collision/reaction cell. The optimized mobile phase was comprised of 50 mM ammonium acetate; 2% methanol, 5 mM myristyl trimethylammonium bromide at pH 4.85 and a C8 column was used. This method provided a highly sensitive baseline separation of the three analytes (ethyl methylphosphonic acid, isopropyl methylphosphonic acid, and methylphosphonic acid) within 15 minutes and detection limits of less than 263 pg/mL, thereby demonstrating a high aptitude for complex speciation analysis of environmental water and soil samples (309). In order to hyphenate IPC-MS to separate and identificate acidic contaminants in ammunition wastes, several types of formates as volatile IPRs were studied. Both tributyl- and trihexylammonium formate proved to be valuable IPRs for the separation of acidic compounds like nitrobenzoic acids, nitrobenzenesulfonic acids and nitrated phenols. Due to the weaker retention of the ion-pairs with trialkylammonium formates compared with tetraalkylammonium compounds, either less organic modifier or a higher concentration of the IPR had to be used. With negative APCI and ESI mass spectrometry it was possible to unambiguously identify several acidic oxidation products of TNT in ammunition wastewater and soil extracts. 2-amino-4,6-dinitrobenzoic acid was often found to be the main metabolite of TNT in such water samples (195). Nitroaromatic and aminoaromatic compounds were determined in concentrations up to few hundred μg/L in ammunition wastewater from the manufacture of nitroaromatic explosives via an IPC method with tetralkylammonium IPRs (432). The complete separation of TNT metabolites via the study of the influence of the temperature and of the presence of an IPR was obtained (238).

Different analytes were monitored in the effluent of potable water treatment plants. Together with trihalomethanes, haloacetic acids can be found in trace amounts in drinking waters as chlorination by-products after disinfection processes. Since they were found to be carcinogenic at low concentrations, their monitoring is increasingly being considered and there is a growing interest in their determination in aqueous compartments. They were determined in different water samples by an analysis method based on SPE enrichment of 50-mL water samples at pH 1.8 followed by IPC-ESI-MS. Complete LC separation of all compounds could only be achieved using triethylamine as volatile IPR. The detection limits were in the low μg/L range. Results from drinking water, swimming pool water, and surface river water samples confirm the ubiquitous occurrence of haloacetic acids in aqueous environments (186). A comparative study was made of the chromatographic behavior of five haloacetic acids (mono-, dibromoacetic and mono-, di-, trichloroacetic acids) using IPC with UV detection, suppressed and non-suppressed anion-exchange chromatography. The advantage of IPC, that can be performed with both tetrabutylammonium–methanol or cetyltrimethylammonium–acetonitrile, was that it did not require the pre-treatment of the sample to remove the interferece of chloride ion. The influence of mobile phase parameters on retention was in agreement with the IPC theory. A pre-concentration step reduced the method detection limits (321). Poly-diallyldimethylammonium chloride (PDADMAC) is a water-soluble cationic polymer widely used as a coagulant in potable water treatment. Its residual concentration in drinking water has been regulated to be 50 μg/L in the USA. A method for the direct detection of diallyldimethylammonium chloride in drinking water treatment plants, using IPC-ESI-MS, was developed and optimized. The chromatographic separation, that did not require any sample preparation, was performed using a C18 column. The pH was adjusted to pH 4.2 to avoid peak tailing and the chosen 5 mM concentration of HFBA, used as IPR, was a trade-off between adequate retention and signal suppression (both increasing with increasing HFBA concentration). The optimized procedure gave good chromatographic retention and sensitivity. Quantification was achieved using selected ion monitoring and the detection limit was 0.1 μg/L. The method was used to detect diallyldimethylammonium chloride in drinking water and its concentration ranged from below quantitation limit to 22.0 μg/L (289).

A method for the trace determination of fluorescent whitening agents in environmental water samples was developed and validated. The analytes were then identified and quantitated by IPC-ESI-MS-MS, applying di-n-hexylammonium acetate as IPR. Limits of quantitation were established between 4 and 18 ng/L in 50 mL of water samples. Precision for these analytes, as indicated by RSD, was less than 13 and 11%, respectively, for intra- and inter-batch. Accuracy, expressed as the mean recovery, was between 68 and 97%. The method was applied to environmental water samples, showing the occurrence of five fluorescent whitening agents in both river water and wastewater treatment plant effluent samples (156).

A similar method was used for the determination of four stilbene-type disulfonates and one distyrylbiphenyl-type fluorescent whitening agent in paper materials (napkin and paper tissue) and infant clothes and applied to commercial samples, showing that two stilbene-type disulfonates were predominantly detected in napkin and infant cloth samples (284).

The previous method was modified by applying fluorescence detection: this did not require a volatile IPR, hence tetrabutylammonium hydrogensulfate was used in the mobile phase. The method was used to determine fluorescent whitening agents in household laundry detergents and surface waters (433). The optimized IPC and CE analyses of stilbene-type fluorescent whitening agents were applied to commercial household cleaning agents and compared: no significant bias was shown between them by t-test at 95% confidence level (317).

IPC methods have been also reported for the analysis of dyes and for the monitoring of their degradation (153, 154, 189, 192, 218, 219, 234, 285, 290, 434). The azo functional group can be found in textile dyes and it plays an important role in dyestuffs chemistry in general. These compounds are highly persistent in the environment. The presence of such dyes in industrial wastewaters is of great concern, because they can produce potentially hazardous products, either by photochemical degradation or via metabolic processes in plants and animals.

The analysis of polysulphonated anionic dyes and their intermediates using IPC-ESI-MS allowed the determination of the molecular mass and the number of sulphonic and carboxylic groups. The most suitable additive as far as compatibility with MS detection was concerned was 5 mM ammonium acetate. However, the retention of compounds with two or more sulphonic groups was too low for a successful separation with ammonium acetate or IPRs with short alkyl chains. The dihexylammonium acetate ion-pairing reagent offered a reasonable compromise in terms of sufficient volatility, adequate retention and separation selectivity for the analysis of polysulphonated dyes (153), but triethylammonium acetate was also profitably used in the analysis of impurities in polysulphonated azo dyes: hyphenation with ESI-MS mass spectrometry made the identification of probable structures of trace impurities in commercial dye samples possible (154).

The effects of the structure and concentration of the ion-pairing reagents on the electrospray response of mono-, di- and tetrasulphonic aromatic acids and acid dyes were studied in detail and it was found that ammonium acetate causes similar signal suppression of (poly)sulphonic acids as di-and trialkylammonium acetates but the separation selectivity is poorer and the ion suppression effect is related to the instrument geometries (189). During the determination of mono- and disulphonated azo dyes by IPC with ammonium acetate as IPR, a comparison between various atmospheric pressure ionization interfaces demonstrated that ESI gave the best performance in terms of sensitivity reproducibility, although the structural information was poor (192).

Metal complex of azo dyes were separated via IPC and monitored via ESI-MS and multistage MS (219) or via electrochemical detector (234).

Electrochemical treatment of wastewaters containing azo dyes in the textile industry is a promising strategy for their degradation. The monitoring of the course of the decomposition of azo dyes in wastewaters is essential due to the environmental impact of their degradation products; it was performed via a gradient elution with triethylammonium acetates IPR; ESI-MS allowed the identification of degradation products electrochemically obtained in a laboratory-scale electrolytic cell in sodium chloride or ammonium acetate as supporting electrolytes (290).

The process monitoring of anaerobic azo-dye degradation was continuously performed via membrane filtration sampling modules and simultaneous IPC separation; the sampling matrix consisted of anaerobic sludge from a municipal wastewater plant (434). The degradation of blue gel pen dyes and changes of blue gel pen ink entries on paper stored in different light conditions and natural environment was studied by IPC-ESI-MS-MS. The chromatographic conditions were optimized by comparing a series of IPRs, including ammonium carbonate, ammonium acetate, triethylamineacetate, tributylamine acetate, tetrabutylammonium bromide and dihexylammonium acetate. It has been found that tributylamine acetate was a suitable ion-pairing reagent for separation of the inks on the common C18 column (285).

Since as an essential requirement for transferring of IPC methods, it is necessary to devise suitable conditions for validation of the methods, system suitability tests based on a group of dyes was developed. A number of parameters was studied, including organic modifier percentage, mobile phase pH, alkylamine chain length, IPR concentration, column temperature, flow rate, and sample composition. Standardized conditions are proposed to assess the suitability of a new column packing material for adequate resolution of the proposed system (435).

Highly soluble benzene and naphthalene sulphonates are widespread organic pollutants since they are widely used in the chemical, pharmaceutical, tannery, textile, and paper industries. The determination of sulfonated and sulphate compounds in waters, wastewaters, and industrial products (190, 191, 194, 225, 267, 279, 288, 436) was the object of many investigations.

IPC-MS-MS determination of aromatic sulphonates in industrial wastewater took advantage of volatile amines as IPR (190). A similar procedure was used for the analysis of sulphophthalimide and some of its derivatives (191), and for the quantitative determination of sulfonated naphthalene-formaldehyde condensates from aqueous environmental samples; in this case fluorescence detection was used for the quantitation and ESI-MS confirmed that the analytes eluted in order of condensation (267).

In the analysis of sulphonated aromatics, to exploit the potential offered by the non-volatile tetrabutylammonium as IPR and to avoid contamination of the ESI or APCI ionization interface, the on-line removal of non-volatile ions by cation exchange suppressor cartridge was devised and successfully used (279).

Tracing polar benzene- and naphthalenesulfonates in untreated industrial effluents and water treatment works was obtained via both fluorescence and ESI-MS: fluorescence gave lower detection limits (sub-ppb range), but ESI-MS provided structural information and unequivocal identification (194, 288).

The IPC of benzene and naphthalene sulfonic derivatives having different charges was optimized as regards to the IPR concentration, the organic modifier percentage in the eluent, and the ionic strength to separate neutral compounds (benzene and naphthalene) from positively and negatively charged analytes; an on-line pre-concentration method, also based on ion-pairing, was developed (225).

The determination of anionic surfactants during wastewater recycling processes by IPC with suppressed conductivity detection was valuable for monitoring their biodegradation because the method did not require any pre-treatment and was free from interference (436).

The separation of aromatic carboxylic acids was performed via IPC in the analysis of coal oxidation products and their retention behavior was studied; linear free energy relationships were observed between the capacity factor and the extraction equilibrium constants of benzoic acid and naphthalene carboxylic acid, while the capacity factor of benzene polycarboxylic acids was proportional to the association constants between aromatic acids and quaternary ammonium ions calculated on the basis of an electrostatic interaction model (142, 231).

Pentafluorobenzoic acid (437) and soil organic phosphorus compounds (438) were determined by IPC.

The separation of ethyl xanthate and its oxidative decomposition products in mineral flotation systems was optimized as regards to the tetrabutylammonium concentration, the eluent pH, and acetonitrile percentage in the eluant. The matrix was particularly challenging because it contained a number of other anionic species, including cyanide complexes of nickel and iron, as well as sulfur-oxy anions (439).

Unusual normal phases such as aminopropyl, cyanoethyl and silica were compared in the determination of alcohol denaturants; the cyanoethyl phase was selected and anionic IPRs were used to reduce rentention of the cationic analyte suppressing their interactions with negatively charged silanols (106).

Enantiomeric Separation

Chiral ion-pair chromatography may offer a valid alternative to chiral stationary phases. Chiral surfactants, first introduced by Terabe in 1989 (440), may give a pseudo-stationary phase (92, 93, 441–444). Ion interaction reagents have also been reported to be useful chiral selectors in the mobile phase when a chiral stationary phase is used (445). This field of research is challenging because chiral stationary phases are not universal and they are very expensive. On the converse, suitable chiral lipophilic ions may provide that three-point interaction with the analyte that is necessary for chiral recognition (446).

There is still a great deal of trial and error in developing a method for the separation of enantiomers. The success of a chiral IPC depends upon the formation of diastereomeric pairs; if they are long-lived they can be separated on an achiral stationary phase since they have different physical-chemical properties. The mechanism underlying each form of separation was presented in a review concerning TLC; this review emphasizes the high sample throughput of TLC, compared to HPLC, and the possibility of using different modes of interaction in the two-dimensional TLC (447). The most important properties of a chiral IPR are: (i) its acid-base behavior, (ii) its hydrophobicity, and (iii) the presence of suitable functional groups (448). Karlsson et al. (449) studied the correlation between computed molecular descriptors from molecular modeling and experimental separation factors in chiral IPC. The best molecular descriptor that explains enantioseparation was found to be the averaged non-polar unsaturated surface area of the complex between the anlyte and the chiral IPR (N-benzyloxycarbonyl-glycyl-L-proline). To sum up, enantioselective separations with chiral IPR on achiral stationary phases are caused by differences in ion-pair formation constants of the two analyte enantiomers with the chiral IPR in the mobile phase and/or differences in the adsorption constants of the formed diastereoisomeric ion-pair to a non-enantioselective stationary phase.

The enantioseparation of amino alcohols was investigated widely via different chiral counterions as (+)-10-camphorsulphonic acid, peptides and N-blocked peptides on different stationary phases (diol-column, porous graphitic carbon, silica gel TLC); studies of the influence of the mobile phase composition, the solute structure, and temperature were presented (92, 93, 101, 102, 240, 450).

The importance of converting the chiral IPR to its dianionic form with sodium hydroxide, regarding enantioselectivity, was underlined when amino alcohols were enantioseparated on hypercarb. Interestingly one diastereoisomer of the chiral IPR gave much more enantioselective retention than the other (92, 93): for example, Z-L-aspartyl–L-proline dissolved in methanol baseline resolved 9 of 12 tested racemates (see Figure 4), but its diastereoisomers, Z-L-aspartyl–D-proline, resulted in low separation factors. Retention decreased with increasing chiral IPR concentration in the mobile phase, with constant ratio between counter ion and sodium hydroxide concentration, because the chiral IPR competes, probably as an ion-pair with sodium ions, with the diastereoisomeric analyte-IPR pairs for the limited amount of adsorption sites. However, decreased retention only slightly affected enantioselectivity because high chiral IPR concentrations improved peak shape. Increased retention and enantioselectivity were observed with decreased column temperature (93) and a column temperature below 0°C was profitably used, on the basis of the van't Hoff plot, when enantiomeric impurities of less than 0.1% were determined even if the enantiomer in low concentration is eluted after the main enantiomer (240). Similarly a low temperature was also used when they were separated via IPC-TLC with camphorsulphonate as IPR (450). The enantioselectivity of animo alcohols with an aromatic ring, that are important beta-adrenergic blocker drugs, decreased in the order orto > meta > para > no substituent, as does the retention, but is only slightly affected by the type of substituent; moreover, a bulky alkyl group attached to the nitrogen atom decreased the enantioselective retention, probably because of the steric hindrance (240).

Successful separations of dinitrobenzoyl derivatives of amino acids performed on a chiral stationary phase using a chiral IPR was investigated (451): this report represents a transition from chiral IPC with chiral IPRs and achiral stationary phases to chiral IPC with achiral IPRs and chiral stationary phases; actually, there are many examples of applications in which achiral IPRs were advantageously used in enantioseparation with chiral colums. For example, chiral chromatography of endogenous neurotoxins made use of· -cyclodextrin as chiral mobile phase additive and heptanesulfonate as IPR (452). The mobile phase for chiral ligand-exchange chromatography for separation of three stereoisomers of octahydroindole-2-carboxylic acid contained the complex of Cu(II) with the optically active selector L-phenylalaninamide and sodium octanesulfonate (453). Moreover, a dramatic and beneficial effect of ethanesulfonic acid (ETA) and particularly methanesulfonic acid (MSA) on the chiral separation of basic compounds on a single chiral column was found. The mechanism appears to be a mixture of ion-pair formation in the mobile phase and increased binding with the chiral stationary phase arising from a localized decrease in pH (386, 454). A diverse set of amine compounds were found to give excellent separations. Since neutral and acidic compounds are unaffected, it can be suggested that maintaining a stable ion-pair is a critical role of the acid additive in the mobile phase. A representative chromatogram of this strategy can be found in Figure 23.

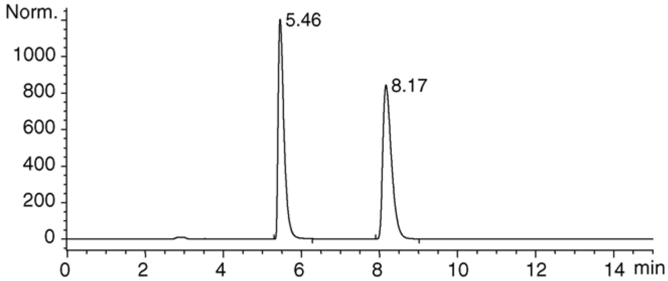

FIG. 23. Chromatogram of ephedrine on an AD-H column using a hexane/isopropanol/ESA (80/20/0.1) mobile phase. Reprinted from ref. (454) with permission from Elsevier.

Tang et al. reported on the beneficial effect of trifluoroacetic acid on the separation of basic compounds in HPLC. (455, 456). Ye et al., during the separation of amino acid esters on Chiralpak AD, observed that when the additives were discontinued, their positive effect did not disappear instantaneously but there was a memory effect (457), thereby indicating that the adsorption of the additive onto the stationary phase is a key factor for enhancing selectivity.

Polarizable chaotropic ions on the Hoffmeister scale are interesting new IPRs especially for the analysis of basic analytes. A series of d,l-dansyl-amino-acids were enantioseparated on a teicoplanin stationary phase. The effect of both a surface tension modifier (sucrose) and a chaotropic agent (perchlorate anion) concentration in the mobile phase was studied. It was demonstrated that the enhancement of the separation factor with increasing the perchlorate concentration was enthalpically controlled, owing to stereoselective bonding interactions (203).

Phenylalanine enantiomers were separated with quinine as the chiral IPR on a normal-phase column (103).

As regards to ion-pairing in different analytical techniques, we underline that several papers have been published in which the chromatographic retention mechanism is said to involve ion-pairing of chiral analites with chiral counter-ions in supercritical fluid chromatography (SFC) with packed columns (328, 458). Ethanesulfonic acid under SFC conditions was demonstrated to act as an ion-pairing agent with a crucial role in the enantioseparation of a wide variety of amines (332).

Chiral counter ions were also advantageously used in enantiomeric separations via ion-pair CE (336, 337, 459–462) (see Figure 16) and ion-pair capillary electrochromatography (463).

Validation

The factors taken into account during a validation procedure are usually the chromatographic run time, the stability of the sample, the linearity of the calibration curve, inter- and intra-day precision and accuracy (relative standard deviation), the limit of detection, the limit of quantitation determination, recoveries, sensitivity, selectivity, and robustness (68, 106, 107, 124, 126, 130, 135, 136, 140, 144, 148, 155, 156, 186, 200, 229, 232, 239, 247, 252, 253, 256, 261, 266, 267, 275, 276, 284, 295, 309, 318, 365, 376, 392, 395, 401–403, 408, 409, 435).

A very good example of the validation procedure of a new method can be found in ref. (132) concerning the dermination of soybean proteins in commercial products by IPC, using a elution gradient. In order to apply the method for quality control purposes, since there is no certified reference material that can be suitably used as a standard for the determination of soybean proteins in commercial products, a commercial soybean flour was used as standard. Claims of the linearity of the model were supported not only by the regression line correlation coefficient, but also by means of the analysis of the residuals and variance. The LOD and LOQ were, respectively, determined from the calibration plot as the concentration corresponding to a signal equal to the intercept plus, respectively, three or ten times the standard deviation of the regression line. The existence of matrix interferences was tested via the comparison of the slopes of the calibration plots obtained by the external and the standard addition calibration methods. The specificity of the new method was demonstrated by regressing added concentration upon recovered concentrations of soybean proteins in commercial products and statistically demonstrating that the slope and intercept did not significantly differ from unity and zero, respectively. The precision of the method was determined by means of the repeatability and of the intermediate precision and quantified by the RSD, %. A recovery test was also successfully performed. The figures of merit of the method are reported in Table 3.

When quaternary ammonium compounds as drugs and herbicides were determined in whole blood the matrix effect study was performed using the percentage matrix effect calculated according to the following expression in which a and b are the peak area ratio of the analyte to internal standard in neat solution and in whole blood:

$$\text{Percentage matrix effect} = (\text{b} / \text{a}) \times 100\%.$$

Since the matrix effect was not neglegible all standard solutions were prepared in whole blood matrix to mimic the actual sample conditions (134).

Matrix effects that occur during ESI may alter the signal intensity of an analyte in the analysis of real samples. In order to detect these effects the response factors obtained from standard addition of the analytes into a wastewater extract (R2) and into pure water (R1) were contrasted. Since the relative response (R2/R1) of the analytes was significantly decreased, their reliable quantification from aqueous environmental samples requires standard addition (143).

The extraction recoveries of risedronate from rat plasma were ascertained by comparing the absolute peak areas of samples spiked with the analyte prior to processing with those of

TABLE 3

Characteristics of the perfusion RP-HPLC method for the analysis of soybean proteins in soybean–wheat and soybean–rice bakery products. Reprinted from ref. (132) with permission from Elsevier

	Soybean–wheat			Soybean–rice
Linear concentration range		Up to 1.30 mg/mL soybean proteins		
Detection limit		0.04 mg/mL soybean proteins (0.10% (w/w))[a]		
Quantitation limit		0.13 mg/mL soybean proteins (0.33% (w/w))[a]		
Existence of matrix interferences				
– Slope by the external standard method		57.1 ± 4.2 (n = 8)		
– Slope by the standard additions method	61.0 ± 2.3 (n = 5)			54.9 ± 2.5 (n = 5)
Specificity[b]		Y = -0.01 (0.02) + 1.04 (0.03)		
Repeatability (RSD,%) (n = 10)[c]	Soybean–wheat sample	Standard		Soybean–rice sample
– Retention time	0.36		0.29	0.33
– Peak area	0.39		0.44	0.17
Intermediate precision[d] (RSD,%)	Standard (0.03 mg/ml soybean protein)			Standard (1.00 mg/ml soybean protein)
– Retention time	1.63			1.03
– Peak area	4.53			8.95
– Slope			9.20	
Recovery (%)[e]				
0.20 mg/ml	99.7			102
0.40 mg/ml	96.5			105
0.60 mg/ml	97.9			103
0.80 mg/ml	98.1			104

[a] Limits of detection and quantitation in percentage (w/w) were determined related to 1.0 g of initial product.

[b] The t-tests for the verification of slope and intercept indicated that they were statistically equal unity and zero, respectively. Standard deviations of slope and intercept are given in parentheses.

[c] Number of injections of a solution of 1.10 mg/mL of soybean flour and of the solutions obtained after solubilization of soybean proteins from a soybean–wheat biscuit and a soybean–rice bread (40.0 mg/mL of initial product).

[d] Analysis performed by the external standard method in 7 days (for retention time and peak area) or in 13 days (for slope) during a period of 7 months.

[e] Recovery obtained for soybean proteins when different amounts of soybean flour were added to the solutions obtained after solubilization of soybean proteins from a soybean–wheat bread and a soybean–rice bread.

drug-free control plasma samples spiked post extraction with suitable concentrations of risedronate (159).

In the IPC-MS analysis of methadone in human plasma, the potential for carry-over from high-concentration analyte levels was evaluated by injecting duplicate extracted matrix blanks immediately after the upper-limit of quantification calibration standards in each validation run (166).

To identify the experimental parameters upon which the response of the method are significantly dependent and to determine the range over which those parameters may vary without affecting the method performance, a fractional factorial design was used to assess the robustness of the the separations of 2,4-disulfonic acid benzaldehyde di-sodium salt-related impurities. It was concluded that the crucial parameter was the organic modifier concentration, followed by buffer pH and buffer ionic strength (113).

CONCLUSIONS AND ANTICIPATED DEVELOPMENTS

The purpose of this review was to make evident and establish the potential and versatility of the IPC strategy that was used, during the last decade, for analyzing a huge range of organic as well as inorganic and organometallic analytes dealing and coping with the most challenging speciation needs in a very short time. Future trends appear to point towards miniaturization, fast and ultra-fast chromatographic runs, efficient and easy hyphenation, and environmentally friendly mobile phases.

The breakthrough of newly introduced chaotropic salts and perfluorinated carboxylic acids as IPRs put a milestone landmark between the present IPC technique and the pristine soap chromatography strategy.

Ion-pair interactions in different analytical fields seem to confer and grant additional versatility and selectivity; it follows that ion-pairing is increasingly being considered, especially in CE and SFC. It is easily predicted that additional theoretical efforts will be required in order to describe and predict analyte behaviors in these new conditions.

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