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Suitability of different C_{18} silica-based stationary phases for the transferability of an Ion-Interaction HPLC method

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Abstract. A critical chromatographic separation of a mixture containing inorganic anions (nitrate and nitrite) and organic cations (the three isomeric forms of phenylendiamine) was chosen as the System Suitability Test (SST) to compare the performance, under Ion-Interaction condition, of five different C_{18} columns characterised by the same specifics.
The ruggedness (*i.e.* the capacity to yield good results also when there are variations in the "experimental circumstances") of the Ion Interaction Reagent (IIR) HPLC method previously developed and optimised was evaluated with robustness tests for each stationary phase considered. Variations of ±10 % to the nominal values of the chromatographic conditions were imposed and the effects on the chromatographic response were evaluated.
For the different stationary phases the results showed performances and behaviours unexpectedly different, concerning not only the resolution among the components of the mixture but also their elution sequence order.
The studies identified the pH value of the mobile phase as the most critical parameter of the method, which must be very strictly controlled. For one of the stationary phases considered a pH variation of only 5 % with respect to the nominal value not only gave unsatisfactory resolutions, but also a different elution order of the analytes.
Another of the tested columns was proved not suitable to work under these IIR conditions.

Keywords. Liquid chromatography – SST – robustness tests – validation.

Introduction

Each analytical method has to be validated, that means that its ability in giving reliable data has to be proved. The validation of HPLC methods is particularly important because they are used on a large scale routinely. When a new HPLC method is developed, the question arises whether the analytical results from the method depend in a critical way on possible small deviations in the mobile phase composition, or in the temperature, or in any variable of the whole experimental condition set.

Beside the so called primary validation process testing accuracy, sensitivity, specificity, precision, etc., recently analysts became more aware of the importance of a second step of validation, that mainly concerns the ruggedness [1]. The ruggedness of an analytical procedure is defined as "its capacity to yield exact results in the presence of small changes of the experimental conditions such as might occur during the utilisation of the procedure", where a "small change" is "any deviation of a parameter of the procedure compared to its nominal value as described in the method of analysis" [2]. These possible deviations of the conditions from the nominal values (as defined in the process of development and optimisation) can not be controlled or avoided because they are due to natural variations of the conditions, and are for instance due to inaccuracy of the analyst, to deviations in the instrumentation performances, to a low stability of the reagents, to variations of the atmospheric conditions of the lab, etc., and in general to all the indeterminate system and laboratory errors which can affect the results of the analysis.

A ruggedness test can be applied to any method that has been optimised, in order to test whether the optimisation process has led to an unstable method [3,4]. A ruggedness study was also defined as "an intralaboratory experimental plan, used before undertaking an inter laboratory study, to examine the behaviour of an analytical process when small changes in the environmental and/or operating conditions are made, akin to those likely to arise in different laboratories" [5]. The secondary validation process represents therefore an essential step for an easy transferability of the method to other laboratories [6].

Generally the tests checking the ruggedness are called "robustness tests" and they try to simulate the variations of the factors that are expected to occur in practice. However, up to now, no uniform ruggedness testing procedure exists and this has led to a variety of approaches proposed by different authors. Molnar reminds us that the specialist brain

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power must be considered as the most important part of the validation step [7].

Of paramount importance is to define which is the response of primary interest that must be tested. Thus, for example, the main aim of a chromatographic method could be to achieve the best resolution between the components of a mixture, or the minimum allowable detection limit, or a short analysis time, etc.

Then the factors to be studied have to be chosen (this selection is mainly based on experience). In fact, an HPLC method can be theoretically influenced by a very large number of factors (about 50 [8]), but probably (and luckily!) just few of them should affect the method performance to an extent that make the results unacceptable.

Among the HPLC techniques, Ion-Interaction Reagent HPLC (IIR-HPLC) is a very versatile one allowing the simultaneous separation of anionic and cationic species. IIR-HPLC is based on the dynamic modification of the surface of the reverse-phase stationary phase by an Ion-Interaction Reagent present in an aqueous or hydro-organic mobile phase flowing in isocratic conditions.

One of the most common theories to explain the retention is the formation of an electrical double layer on the surface, able to exchange both anions and cations [9]. In the last years in our lab IIR-HPLC methods making use mainly of alkyl ammonium phosphate as IIRs have been developed. The retention has been shown to be influenced by a number of factors such as the alkyl chain length of the IIR, the concentration of IIR and the amount of organic modifier in the mobile phase, the mobile phase pH, the temperature, etc. [10-12]. This means that the technique is highly versatile and able to solve separation problems not possible by traditional RP-HPLC, but there is also a larger number of factors to be considered in a ruggedness study.

In this work, a possible approach to study the ruggedness of an Ion-Interaction HPLC method is presented. In particular the separation of a mixture including inorganic anions (nitrite and nitrate) and organic cationic species (three isomers of phenylendiamine) was studied. The problem is begun by considering the effects of imposed ±10 % variations to the "nominal" value of the experimental parameters. The robustness has been studied also with regards to the transferability of the method to different stationary phases: five silica-based RP-18, 5 μm, end-capped columns made by different manufacturers have been considered.

In the ruggedness study, a system suitability test (SST) was developed, which considers the whole system including all the steps involved. Usually in chromatography the separation of a mixture containing analytes developed with critical resolution criteria could be a good SST of a "visual" type.

The resolution of the mixture discussed here can constitute a good System Suitability Test. It can be run each time it is necessary to check the method for continued suitability for use with different systems [11,12].

Experimental

Apparatus

The analyses were carried out with a Merck-Hitachi Model L-6200 LiChrograph chromatograph (Tokyo, Japan) equipped with a two channel Merck-Hitachi Model D-2500 Chromato-integrator. The chromatograph was interfaced with a Model L-4200 UV-Vis detector and was equipped with a LiChrograph oven with temperature control.

A Metrohom 654 pH-meter (Herisau, Switzerland), equipped with a combined glass-calomel electrode was employed for pH measurements.

The stationary phases considered were five types of commercial C_{18} columns and namely: Spherisorb ODS (Phase Separation) 250 mm × 4.6 mm, 5 μm, 14 % C; Allsphere (Alltech) 250 mm × 4.6 mm, 5 μm, 12 % C; Nucleosil (Alltech) 250 mm × 4.6 mm, 5 μm, 14 % C; LiChrospher (Merck) 250 mm × 4.6 mm, 5 μm, 18 % C; Purospher (Merck) 125 mm × 3.0 mm, 5 μm, % C not given. All the considered packing materials have a regular spherical shape and are fully end-capped.

Reagents

Ultrapure water from a Millipore Milli-Q system (Milford, MA) was used for the preparation of all the solutions. Heptylamine, o-phosphoric acid and 1,2-, 1,3-, 1,4-phenylendiamine (1,2-, 1,3-, 1,4- PA) were Fluka (Buchs, Switzerland) analytical grade chemicals. Acetonitrile, sodium nitrite and sodium nitrate were Merck (Darmstadt, Germany) analytical grade chemicals.

Chromatographic conditions

The mobile phases were prepared by adding diluted o-phosphoric acid to the heptylamine aqueous solution of the desired concentration to the prefixed pH.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was reached and when reproducible capacity factors were obtained for three subsequent injections.

Spectrophotometric detection at 230 nm was employed.

Results and discussion

As mentioned, the simultaneous separation of inorganic anions (NO_3^- and NO_2^-) and organic cations (the three isomeric forms of phenylendiamine) was chosen to study, under IIR conditions: i) the performance of the stationary phases considered and ii) the ruggedness of the method when transferred onto the different columns. This IIR-HPLC separation can be considered a good SST, because the resolution is quite critical and the retention of the analytes is differently affected by the chromatographic variables: for instance, changes of *pH* and of ion-interaction reagent concentration

C_{IIR} in the mobile phase would have opposite effect on the retention of anions and cations.

The chromatographic method has been previously developed in our laboratory and the conditions optimised by chemometric treatment of experimental design [13] to reach the maximum resolution among the analytes in the shortest total analysis time. The method, that will be called throughout this paper the "reference method", employed a Spherisorb ODS-2 as the stationary phase and a 6.6 mM heptylammonium aqueous solution (pH = 6.4) as the mobile phase (flow-rate = 1.0 mL/min).

The ruggedness of the method has been studied by imposing ± 10 % variations to the nominal value of the following factors: mobile phase *pH*, flow rate *f*, IIR concentration C_{IIR} and temperature *T*, being a variation of the 10 % usually larger than those which can naturally occur.

The robustness for these variables was evaluated for the different RP stationary phases and the results were compared, finding some interesting and unexpected results.

Spherisorb ODS-2 stationary phase

The first step to test the robustness of the RP-IIR-HPLC method chosen as the SST was to replicate the "reference" method by using the same commercial packing material used in its development and optimisation [14] but in the cartridge form now available (and obviously from a different batch). The replicability was really poor: the chromatogram obtained showed much shorter retention times and a general unsatisfactory resolution of the analytes. The reason of this irreproducibility is difficult to understand (which might be ascribed to a quite different packing material). From information gathered in the meantime, it appears that the manufacturer of the Spherisorb stationary phases has changed.

Since these results were so dramatically different from those expected, no attempt was made to adjust the method for this newer stationary phase, but another one was tested.

Allsphere ODS-2 stationary phase

The separation obtained on this column was, on the contrary, quite satisfactory. Moreover with an adjustment of the pH (from 6.4 to 7.0) and of the flow-rate (from 1.0 to 0.7 mL/min) a resolution better than that of our "reference method" was obtained, as shown by the chromatogram reported in figure 1a. Retention time reproducibility was still within 3 % for the same preparation of mobile phase and within 6 % for different preparations. These conditions are therefore chosen as the new "nominal conditions".

It should be noted that the elution sequence order is different, being NO_2^-, 1,4-PA, NO_3^-, 1,3-PA and 1,2-PA for the Spherisorb column [13] and NO_2^-, NO_3^-, 1,4-, 1,3-, 1,2- PA for the Allsphere.

To test the ruggedness of the method for this stationary phase, variations of ±10 % were imposed to the *nominal values* of the experimental parameters, which now are: 6.6 mM of octylamine at pH = 7.0 as the mobile phase, flow-rate of 0,70 mL/min. ±10 % variations are reasonably greater than those that can naturally occur during the application of the method and are therefore suitable, if the tests are positive, to assure the robustness of the method. The resolution of the five analytes is the response that must be checked.

Table I. Per cent variations in the retention times of the studied analytes as a function of ± 10% variations of concentration of ion-interaction reagent (C_{IIR}), pH and flow-rate (f).

	NO_2^-	NO_3^-	*1,4-PA*	*1,3-PA*	*1,2-PA*
C_{IIR} + 10%	-2.38	-1.87	-12.51	-10.67	-10.74
pH + 10%	-14.14	-14.95	-13.98	-13.68	-14.39
f + 10%	-10.14	-10.47	-10.94	-10.67	-10.67
nom					
f - 10%	10.89	10.70	10.46	10.37	10.75
pH - 10%	5.92	4.23	-9.23	0.21	-0.15
CIIR - 10%	-1.97	0.40	-5.08	-3.31	-2.97

Stationary phase: Allsphere (Alltech) 250 mm × 4.6 mm, 5 µm, 12 % C
Nominal conditions of mobile phase: 6.6 mM of octylamine at pH = 7.0 (o-phosphoric acid), flow-rate of 0.70 mL/min

The results obtained are reported in table I as the % variations in the retention time, induced by the new intentionally-varied conditions, with respect to the times obtained under the nominal conditions.

As expected, the flow-rate variations are always of the same order of the variations imposed (±10 %), and indicate that the instrumental performance is under control. For the other variables it is impossible to ascertain a trend concerning both the absolute value and the sign. This behaviour is due to the different processes that govern the retention mechanism in ion-interaction chromatography and, as expected, a different behaviour can be envisaged for cationic and anionic species for variations of both C_{IIR} and *pH*.

As concerns the resolution, this was always maintained for the ±10 % variations imposed. The analytical method chosen as SST can be therefore considered robust when transferred to the Allsphere column.

LiChrospher 100 RP 18 Merck

Figure 1b shows the chromatogram obtained when the method was applied on a LiChrospher 100 RP 18 Merck column, under the nominal conditions. As it can be seen, the total analysis time is comparable with that obtained for the Allsphere column and the resolution is maintained even if it becomes critical between 1,3-PA and NO_3^-, (Fig. 1b). The most surprising result is the inversion of the sequence order, with respect to the Allsphere column; the sequence is 1,4-PA, NO_2^-, 1,3-PA, NO_3^-, 1,2-PA and is the same observed for the Spherisorb column in the originally developed method.

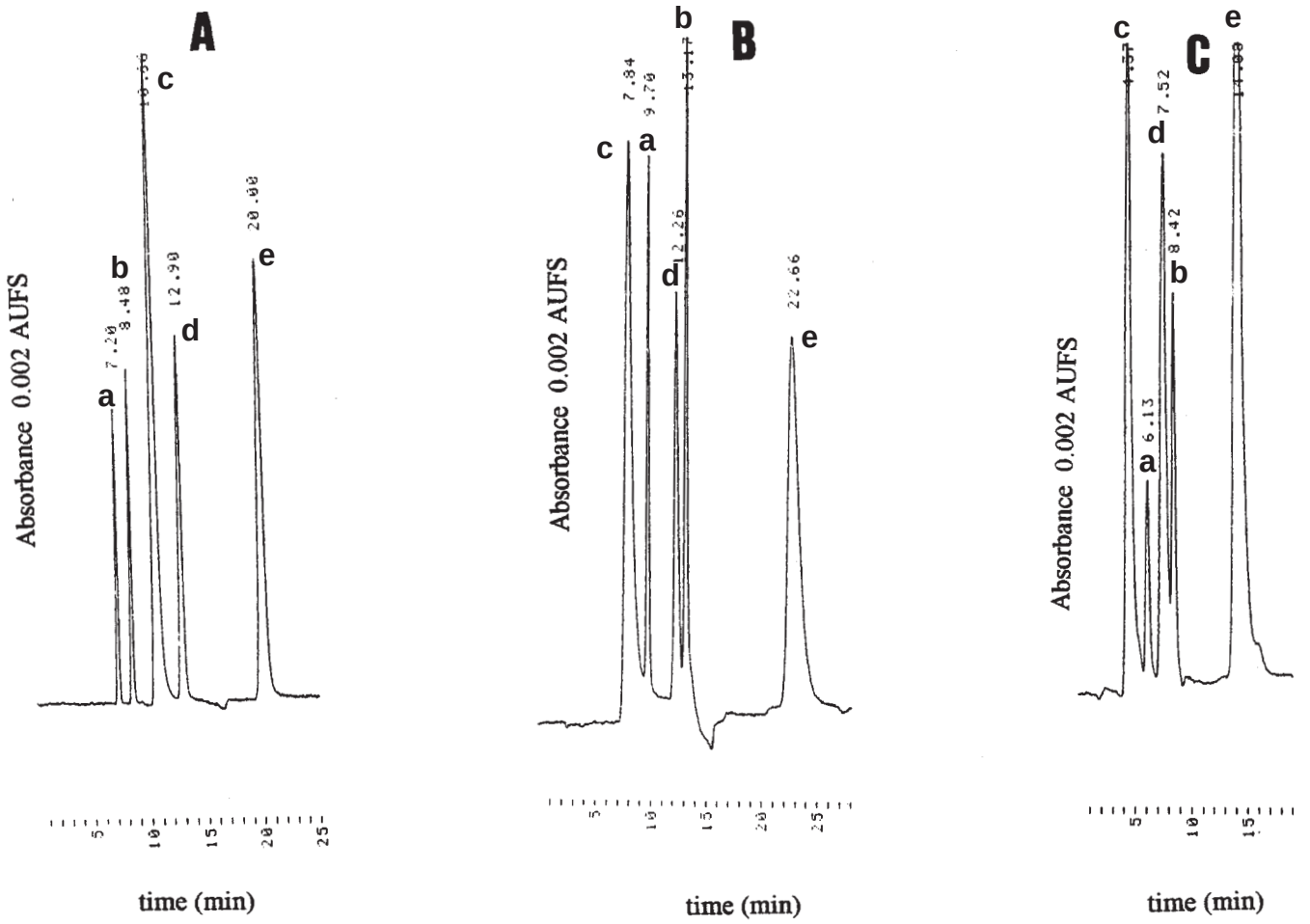

Fig. 1. Chromatographic separation of the mixture in "nominal" conditions: a) nitrite, b) nitrate, c) 1,4-PA, d) 1,3-PA and e) 1,2-PA (0.10 mg/L each).
Stationary phases:
a) Allsphere (Alltech) 250 mm × 4.6 mm, 5 µm, 12 % C
b) Lichrospher (Merck) 250 mm × 4.6 mm, 5 µm, 18 % C
c) Purospher (Merck) 125 mm × 3.0 mm, 5µm, % C not given
Mobile phase: 6.6 mM of octylamine at pH = 7.0 (o-phosphoric acid), flow-rate of 0,70 mL/min.

The results obtained for the LiChrospher stationary phase in the retention times for ±10 % variations in the values of the variables of C_{IIR}, pH and f are reported in table II.

While the effect of flow-rate on the retention times is, as for the Allsphere column, proportional to the variations induced, the effects led by the variations of C_{IIR} and *pH* are greater than those observed for the Allsphere column. In particular, *pH* variations play their role in the same direction but variations are greater, so that *pH* becomes a critical parameter and variations of ±10 % lead to a loss of resolution. In addition, both 10 % *pH* increase and decrease lead to an inversion in the elution sequence order between 1,3-PA and NO_3^-.

The method was therefore tested for only ±5 % *pH* variations (Fig. 2 d,e): the resolution was maintained for 5 % negative variations but not for 5 % positive variations; the elution sequence order is as that observed for 10 % variation. A test performed for ±2.5 % variations indicated that the method is robust to both these variations and furthermore no inversion in the elution sequence order takes place.

As a conclusion, it can be said that in the use of the LiChrospher column the critical variable which must be carefully checked in the preparation of the mobile phase, is the pH value, mainly as concerns possible positive errors. Deviations of only ±2.5% are allowed.

Nucleosil stationary phase

Unsatisfactory results were obtained for the Nucleosil stationary phase. Besides the coelution observed between 1,3-PA and NO_3^-, generally broad and asymmetric peaks were

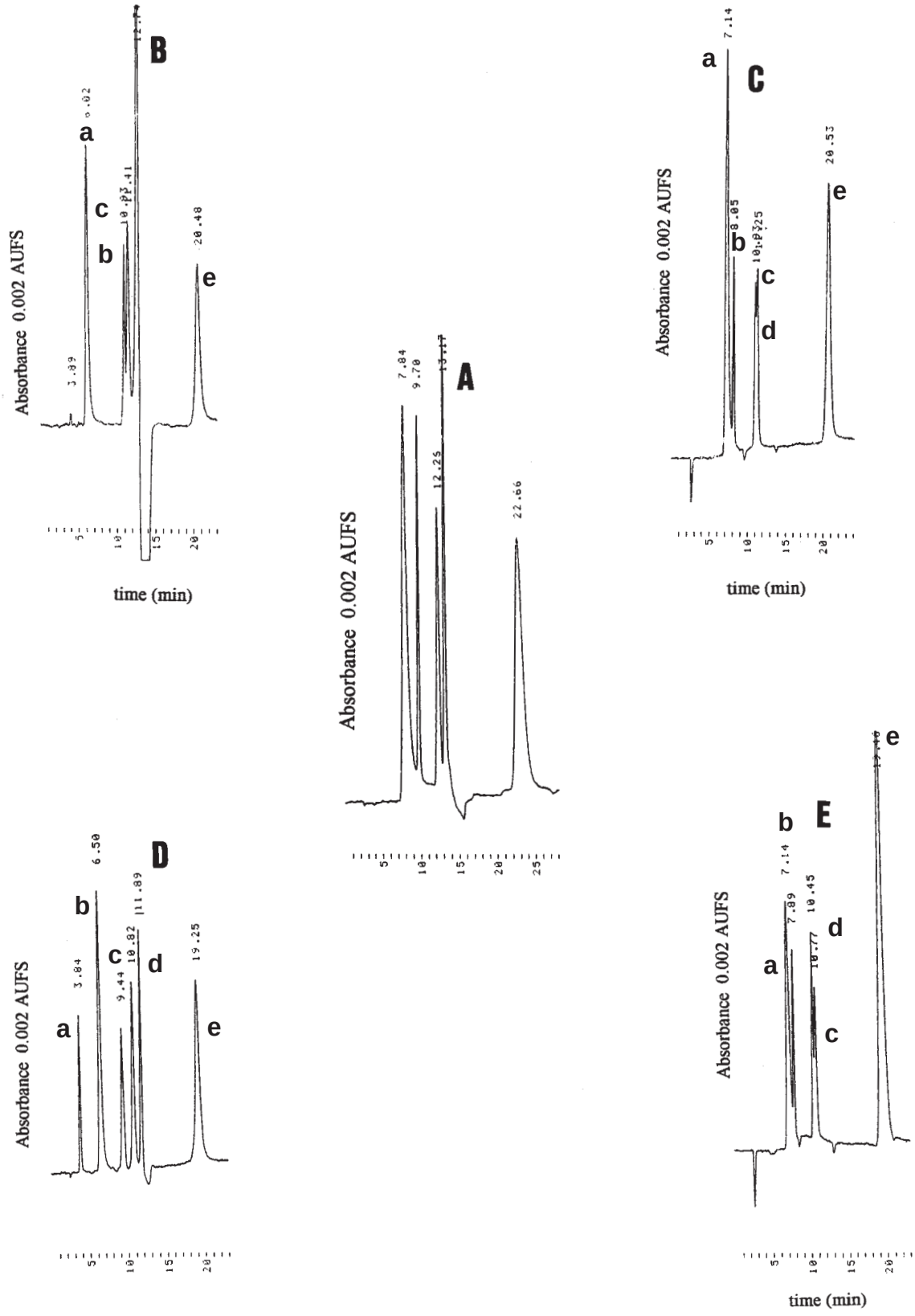

Fig. 2. Lichrospher stationary phase. Influence of the mobile phase pH variations on the resolution of the mixture.
a) nominal conditions: 6.6 mM of octylamine at pH = 7.0 (o-phosphoric acid), flow-rate of 0,70 mL/min; b) –10 % pH , c) +10 % pH, d) –5 % pH , e) +5 % pH.

obtained. On the other hand the test separation in RP-mode of uracil, phenol and toluene reported in the column leaflet (H_2O-ACN, 42:58 v/v as the mobile phase) could be easily reproduced. It seems that this material packing is not suitable to work in ion-interaction reagent mode. More difficult to try to explain why, taking into account that all the characteristics, including the declared per cent carbon load, are very similar to the other stationary phases investigated.

Purospher RP18

This stationary phase is characterised by the same parameters as the other here tested, a part the length which is 12.5 cm instead of 25.0 cm. Therefore the *f* was halved with respect to the nominal one (0.35 mL/min instead of 0.7 mL/min). Under conditions of mobile phase of C_{IIR} 6.6 mM, pH = 7.0 and f = 0.35 mL/min, a good resolution was obtained as shown in figure 1c; the elution order is the same as obtained for the other Merck column (LiChrospher). Lowering the flow-rate keeps the time the same but efficiency increases since linear velocity changes. The results obtained for the robustness tests carried out for variations of ±10 % of the variables C_{IIR}, pH and f are reported in table III. The behaviour is similar to that obtained with the LiChrospher column, being pH the critical variable. In particular the resolution is lost for 10 % increase of pH, while is maintained for 10 % decrease. Variations of ± 5 % in the pH value of the mobile phase does not lead to changes in the elution sequence and give good resolutions. In a whole, the method transferred onto the Purospher column results more robust to pH change than when using the LiChrospher column.

Temperature effect

All the experiments described up to now were performed at controlled temperature of 25.0 ± 0.5 °C. The ruggdness of the method was also studied for changes of temperature on the Allsphere column, which seems to give the most robust method.

The T range investigated was from 25 °C to 50 °C with 5 °C steps. The measurements were performed after 60 min equilibration times. As expected, retention times decrease when temperature increases. The resolution of the analytes is always maintained in the whole range studied.

Conclusions

The experiments performed to evaluate the robustness of the method chosen as SST, clearly show how difficult it can be to implement of a literature method.

First of all, the importance of the commercial stationary phase packing, even when characterised by the same parameters, was evidenced. The most surprising result concerns the different elution order of the components of the mixture that can be observed for the different columns, that can lead to wrong peak attribution with the lack of a selective detector.

One possible explanation could be the presence of non endcapped silanol groups, which can intervene in the retention process, mainly when electrostatic forces play a role and ion-exchange or ion-interaction equilibria intervene. This hypothesis could be supported by the different elution sequences observed as a function of the pH value of the mobile phase. Inorganic anions and organic amines behave differently both concerning electrostatic interactions and pH induced effects.

Concerning resolution and robustness, the five packing materials investigated and, even characterised by the same parameters (a lower difference concerned the %C load), showed different behaviours. Only one column is robust to all the experimental conditions at the imposed level of ±10 %. Two stationary phases turned out to be unsuitable for the method tested. The remaining two gave good resolutions

Table II. Per cent variations in the retention times of the studied analytes as a function of ± 10 % variations of concentration of ion-interaction reagent (C_{IIR}), pH and flow-rate (f).

	NO_2^-	NO_3^-	*1,4-PA*	*1,3-PA*	*1,2-PA*
C_{IIR} + 10%	-12.39	-11.89	-14.56	-17.34	-18.15
pH + 10%	-17.49	-17.80	-10.75	-9.65	-10.92
pH + 5%	-19.14	-21.30	-10.96	-13.20	-15.41
f + 10%	-8.90	-9.91	-15.38	-11.51	-12.70
nom					
f - 10%	13.64	13.32	4.00	10.77	9.92
pH - 5%	-3.25	-10.18	-18.50	-12.23	-16.04
pH - 10%	12.26	-4.09	-24.75	-7.56	-10.76
CIIR -10%	-6.74	-7.09	-11.79	-12.80	-13.86

Stationary phase: Lichrospher (Merck) 250 mm × 4.6 mm, 5 µm, 18 % C
Nominal conditions of mobile phase: 6.6 mM of octylamine at pH = 7.0 (o-phosphoric acid), flow-rate of 1.0 mL/min

Table III. Per cent variations in the retention times of the studied analytes as a function of ± 10% variations of concentration of ion-interaction reagent (C_{IIR}), pH and flow-rate (f).

	NO_2^-	NO_3^-	*1,4-PA*	*1,3-PA*	*1,2-PA*
C^{IIR} + 10%	0.60	3.88	-5.02	-3.38	-1.68
pH + 10%	15.74	-14.92	-5.41	-10.37	-10.66
pH+5%	-3.84	2.36	-2.47	-3.88	-1.68
f + 10%	-8.38	-9.13	-8.38	-9.72	-9.75
nom					
f - 10%	11.82	11.30	12.07	10.84	10.82
pH - 10%	5.60	12.80	-14.36	-1.71	-1.44
CIIR -10%	-0.44	-0.67	-0.27	0.19	0.53

Stationary phase: Purospher (Merck) 125 mm × 3.0 mm, 5 µm, % C not given
Mobile phase: 6.6 mM of octylamine at pH = 7.0 (o-phosphoric acid), flow-rate of 0.35 mL/min.

under the nominal conditions but were not robust to pH variations of ±10 %. In general, the pH value of the mobile phase was the variable that must be more carefully controlled and checked. This result, on the other hand, is not surprising, taking into account that in the ion-interaction mode the mobile phase pH plays an important role not only on the acidic dissociation equilibria of the analytes but also on the extent of the modification induced onto the stationary phase surface. pH variations in fact affect the molar fraction of the protonated alkylamine which is adsorbed as the first electrical layer onto the surface of the stationary phase.

It must be pointed out that in the chromatograms recorded for all the stationary phases studied (see figures 1 and 2), a dependence of the sensitivity on the different experimental conditions can also be noticed. This behaviour was already observed in previous studies in ion-interaction mode [14,15] and has not yet been explained.

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