CONTRIBUTIONS TO THE DEVELOPMENT OF OPTIMISATION METHODOLOGIES IN LIQUID CHROMATOGRAPHY

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1. Introduction

Liquid chromatography (LC) has spread out since the 70s in analytical laboratories, due to its versatility, ease of use, robustness, sensitivity and applicability to multiple problems in environmental, pharmaceutical, clinical and food analysis. Reversed-phase liquid chromatography (RPLC) is the most common LC mode for the separation of non-volatile compounds in a wide polarity range, from small molecules to large biological macromolecules [1]. However, in comparison with gas chromatography, it has the disadvantage of insufficient selectivity to achieve adequate resolution in the analysis of complex samples, due to its low efficiency. Also, the selectivity and analysis time depend in a complex way on several experimental factors that interact with each other, such as the organic solvent content, pH or temperature, which in practice are difficult to adjust to optimal levels.

The goal of chromatographic optimisation is to obtain the highest resolution in an acceptable analysis time. This process is often carried out by trial and error, which will hardly lead to the true optimum, and the search will often require long experimental work. This is the reason for the development of interpretive optimisation methods, based on the knowledge of the system through mathematical models [2,3], from which the prediction of the separation under multiple experimental conditions is possible. With proper separation strategies and mathematical tools, the best conditions can be found in convenient times. The analyst's effort will mainly be focused to develop the best model describing the retention.

2. Start of the optimisation research line in the FUSCHROM group

In 1989, we started a research in the field of the analysis of banned drugs in sport with Guillermo Ramis-Ramos, as a result of the celebration of the Barcelona Olympics, for which we proposed the use of an LC technique still with little development, called Micellar Liquid Chromatography (MLC) [4,5]. MLC is an RPLC mode, where the mobile phase contains surfactant micelles and a relatively small amount of organic solvent. Its main appeal is the possibility of making the direct injection of physiological samples into the chromatograph. However, we quickly became aware of the difficulties of performing the simultaneous optimisation of the two or three factors involved in method development (usually, the concentrations of surfactant and organic solvent), to which the pH could be added [4,6].

Trying to find a solution to reduce the experimental work that such optimisation involved, an article fell into our hands where the authors applied an optimisation strategy, called the "triangle method". In this method, the experimental domain was split into subspaces, and for each one, a linear model was obtained to predict the retention in other conditions inside the subspaces [7]. At that time, José Ramón Torres-Lapasió was starting his PhD. in the field of MLC, and searched solutions for the optimisation of analytical methods. This was also the start of a research line and the formation of a group, working on the fundamental development of LC (especially in the pharmaceutical field), and chemometrics focused on modelling, optimisation, peak recognition and deconvolution, together with multivariate techniques [3].

We have also applied the methodologies to increase the knowledge on solute-stationary phase interactions (at the thermodynamic and kinetic levels), in the absence and presence of secondary equilibria, using chromatographic columns operating with different chemistry, based on partitioning or with mixed behaviours. The research has been applied mainly to conventional and fast LC, but also to capillary/nano-capillary LC, and capillary electrophoresis. In the investigations, the work developed by other members of the FUSCHROM group should be also highlighted: Juan José Baeza-Baeza, María José Ruiz-Angel and Samuel Carda-Broch. Notable collaborations have been established with Desiré L. Massart (Vrije Universiteit Brussel, Belgium), Elisabeth Bosch and Martí Rosés (Universitat de Barcelona, Spain), Gabriel Vivó (University of Amsterdam, Netherlands), and Sergio López Ureña (Department of Mathematics, Universitat de València, Spain).

3. A magical mystery tour

In the 60s, the "mystery tours", in which only the driver knew the final destination, were common in Britain. When we face a new chromatographic problem, we also have this feeling. Fortunately, nowadays, different chemometric tools are being developed to assist the chromatographer in the exhaustive exploration of the possibilities offered by the separation system. These tools allow to make a magical mystery tour inside the chromatographic system [8].

The FUSCHROM group has been involved in finding the solution to problems in LC at different levels. As commented, the first objectives were related to the optimisation of isocratic conditions in the presence of secondary equilibria, involving surfactants, and more

recently, ionic liquids. This allowed the search of the most convenient organic solvent and additive to improve the separation performance in a given sample, and the comparison of the performance of columns containing stationary phases from different manufacturers, or with different nature (conventional, submicro, silica-based or polymeric monolithic columns). Very extensive work has been carried out regarding the comparison of the accuracy of retention models with one to three factors [2], for a wide range of compounds, in hydro-organic RPLC, MLC, submicellar LC, microemulsion LC, and hydrophilic interaction liquid chromatography (HILIC).

In the RPLC analysis of samples containing solutes in a wide polarity range, with conventional hydro-organic eluents, a gradual increase in the modifier content (a gradient) is needed to obtain good resolution in adequate analysis times. We have developed methodologies to optimise different types of gradients (multi-isocratic, linear, or multi-linear) to accommodate the separation needs of a sample. The increase in the complexity of the gradient program up to an optimal level, by inserting more linear segments, has been also considered [9]. The reliable prediction of gradients requires more complex algorithms, with longer computation time. This handicap has been solved by developing more powerful tools as the multi-scale optimisation of gradients [10], where the level of detail is increased along the search, and using root-finding methods for solving the fundamental equation for gradient elution, which can decrease the computation time in one or more orders of magnitude.

A reliable optimisation of the resolution not only needs a good model to predict the retention, but also an accurate description of the peak profile. It should be noted that chromatographic peaks are often non-Gaussian, and their width is significant with regard to peak distance. Peak profiles are the final result of different types of interactions within the column (partitioning, adsorption/desorption, ion-exchange and size exclusion), to which diverse intra-column effects (axial dispersion and diffusive migration through the packing pores), and extra-column dispersion (within the connecting tubing and with other components in the equipment) should be added. The FUSCHROM group proposed in 1997 a modified peak model for LC that interprets the deviations from an ideal Gaussian peak as a change in the standard deviation with time, according to a polynomial function [11], which has been qualified as the most accurate by several authors. We proposed later other non-Gaussian peak models to adapt them to diverse situations related to the prediction and deconvolution of peaks. The simulation of peaks is carried out based on the prediction of the left and right peak half-widths. For this purpose, we have used several approaches, as the use of mean efficiencies, the interpolation of efficiencies in subspaces close to the predicted condition, and the development of models describing the variation of half-widths versus the retention times [12], which in turn are predicted as a function of the experimental conditions.

4. Experimental designs and COFs

The goal of an interpretive optimisation is finding the experimental conditions that provide maximal resolution (Fig. 1), using the data from a set of experiments as reduced and informative as possible. The reliability of the optimisation will depend, in the latter term, on the quality of the experiments used to build the retention models. Hence the importance of developing an adequate experimental design, which should be obtained with minimal effort. Owing to the number of involved variables, the search of the best design is more complex in gradient elution compared to the isocratic mode.

Recently, we have proposed a method to evaluate in silico the performance of training experimental designs composed of isocratic or gradient runs, oriented to the fitting of retention models, which further will be used to predict retention times in both isocratic and gradient elution [13]. The method, based on the error propagation theory, is able to evaluate the effect on the prediction performance, when several parameters in the designs (number and distribution of the experiments, initial and final modifier content, gradient slope(s), and location of gradient nodes) are varied. We have also reported an alternative proposal to gradient experimental designs, where sudden transient increases of organic solvent are inserted in the weakest experiments of isocratic designs. We have been also involved in the transference of data between isocratic and gradient experiments, and between columns, and in obtaining the significant concentration ranges in gradient elution.

Interpretive optimisation implies the evaluation of the performance of thousands of computer-forecasted chromatograms. In the simplest cases, only the location of the peaks is considered, but the accurate simulation of a comprehensive signal versus time will enhance the reliability. Each simulated chromatogram corresponds to a particular set of experimental factors, which are wisely tuned throughout the optimisation. The exploration may be carried out visually, but with the assistance of a chromatographic objective function (COF), the examination will be more exhaustive [2,14].

A COF is a mathematical expression that qualifies the resolution level associated with any peak distribution. It may include additional quality criteria, such as analysis time, retention of the first and last peaks, solvent consumption, or number of resolved peaks. However, often it only considers the resolution. We have demonstrated that the peak purity, which measures the area free of overlapping for each peak, is closer to the resolution appraisal of an expert analyst than other COFs. This criterion also provides a realistic evaluation of the separation based on the full signals, and is a normalised measurement that qualifies individual peaks, instead of peak pairs [15].

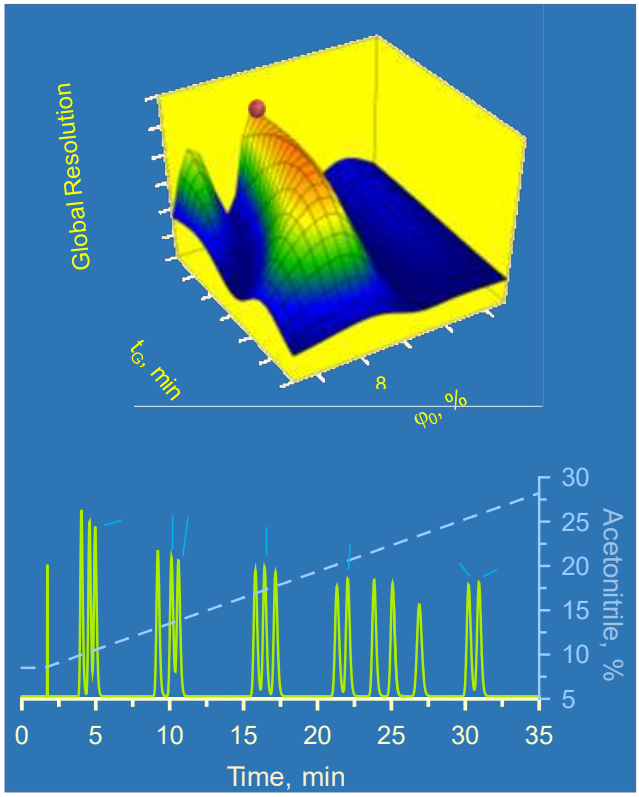

Figure 1. Optimisation of the resolution of 16 β-blockers, in gradient elution, with acetonitrile-water at pH 3, using the peak purity criterion (t_G = gradient time, and φ_0 = initial ramp modifier content). The optimal conditions are marked in the resolution diagram (the corresponding chromatogram is given).

This facilitates a comprehensive inspection of the compounds of interest, and the inclusion of other quality criteria and peak weighting, or even, the exclusion of peaks. All these features have led to the proposal of new optimisation strategies, based on information not accessible using other COFs. The development of a COF based on peak counting [16], useful for situations of extremely low resolution, and the extension of the peak purity criterion to three-dimensional spaces are relevant. In all these searches, the capability of anticipating the consequences of departing slightly from the ideal optimal conditions (robustness) is important [17]. We have also proposed a COF to include both time and spectral information, when the chromatographic order is not capable of getting enough resolution.

5. Analysis of complex samples

Throughout the years, the FUSCHROM group has been interested in solving increasingly complex samples and problems, for which new solutions have been proposed. Conventionally, standards are needed to build the retention and half-width models. We have recently become interested in the possibility of optimising complex samples for which no standards are available, which are analysed by obtaining their chromatographic fingerprints [15]. Since pattern recognition requires obtaining chromatograms that show the maximal number of peaks, we have been working on the possibility of optimising these separations using chemometric methods.

Initially, we developed a COF similar to peak purity that allowed the direct appraisal of resolution in fingerprints, without the need of standards, which we called peak prominence [15]. We also improved a baseline subtraction procedure adequate for highly complex multi-analyte signals, and designed an approach for the measurement of the peak capacity, based on peak simulation, which can be applied to any type of gradient [18]. Finally, we developed an approach to build a global retention model that allows predicting the peaks for complex chromatograms, in the absence of standards [19].

For the search of the best experimental conditions, contour maps and three-dimensional plots have been used (Fig. 1), as well as Pareto plots that consider both the resolution and analysis time. For the most complex cases, search strategies based on natural computation have been developed.

Chromatographic problems are usually addressed trying to optimise a single experimental condition, at which all compounds in the sample are resolved. When the separation fails, the usual choice is introducing a drastic change in the chromatographic system (column, solvent, pH, temperature, etc.). There are, however, other possibilities that do not need new experiments, based on the concept of complementary situations (isocratic mobile phases, gradients, columns, or chromatographic modes). They refer to the optimisation of two or more separation conditions that optimally cooperate in the resolution. One condition is focused on the resolution of a group of compounds in the sample, while the other compounds are resolved using a second (or subsequent) condition(s). However, this idea still presented challenges in terms of computation volume and complexity of the required algorithms, in which we have been working.

On the other hand, there is a belief that the optimisation of selectivity in LC is dominated by the mobile phase. Therefore, a column (with a given stationary phase, length and other specific characteristics) is selected to perform the analysis. However, this does not often succeed for complex samples, and the analyst is forced to change to another column, which can also be unsuccessful. A relatively simple solution to enhance chromatographic resolution is the modulation of the stationary phase through the serial coupling of columns using compatible elution conditions. For this purpose, two or more columns containing different stationary phases and lengths are connected [20]. The combination of columns has the same effect as having a stock of tens, hundreds, thousands (or even more) columns in our laboratory. Each coupled column behaves like a new one with new selectivity expectations and limitations. However, to exploit this possibility, it was necessary to develop optimisation strategies and chemometric tools, which has been an active field for our group in the last

10 years. More recently, we have been interested in applying our developments to optimise the column combination and experimental conditions in two-dimensional LC. The multi-column approaches may represent a stimulus for the proposal of new procedures, especially in combination with mass spectrometric, electrochemical and refractometric detection.

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