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Predictive kinetic optimisation of hydrophilic interaction chromatography × reversed phase liquid chromatography separations: Experimental verification and application to phenolic analysis

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ABSTRACT

Method development and optimisation for comprehensive two-dimensional liquid chromatography (LC × LC) is complex, since this involves the intricate relationships between a large number of experimental parameters with the aim of achieving three conflicting goals: maximising separation performance (peak capacity), minimising analysis time and minimising dilution. This is especially true for the on-line combination of hydrophilic interaction chromatography (HILIC) and reversed phase LC (RP-LC) due to the relative elution strengths of the mobile phases used in these modes, which has severe implications for the modulation process and dilution.

In this study we report a predictive kinetic optimisation tool for on-line HILIC × RP-LC which is based on theoretical relationships between the optimisation goals, the target analyte properties and chromatographic parameters (column dimensions, flow rates, mobile phases, injection volumes, etc.), allowing all chromatographic parameters to vary simultaneously within defined ranges. Experimental restrictions, such as pressure limits, flow rates, etc., are enforced to ensure all results are practically achievable on a given instrumental configuration. A Pareto-optimality approach is then used to obtain optimal sets of experimental conditions, from which the one(s) best satisfying the requirements in terms of time, dilution and/or peak capacity can be chosen. To overcome the challenges associated with mobile phase incompatibility in the coupling of HILIC and RP-LC, splitting of the first dimension HILIC flow, dilution with an aqueous make-up, or a combination of both, were investigated to establish the best approach to minimise total dilution and maximise performance. The validity of the methodology is demonstrated by deriving optimal conditions for the HILIC × RP-LC separation of procyanidins on selected columns and subsequent experimental verification of the performance for the separation of a cocoa extract.

1. Introduction

On-line comprehensive two-dimensional liquid chromatography (LC × LC) is a powerful separation technique that can be used to partially overcome the performance limitations of one-dimensional (1D) LC systems [1–3]. The increased performance of LC × LC comes at the cost of more expensive and intricate instrumentation, while method optimisation is also much more complex than is the case for 1D LC. This is because optimisation in LC × LC requires consideration of a large number of experimental parameters and their effects on achieving three conflicting goals: maximising separation performance (peak capacity), minimising analysis time and minimising dilution. Method development in LC × LC has therefore been the focus of several important papers in the last decade.

The first protocol that comprehensively covered most experimental parameters relevant in LC × LC method development was reported by Schoenmakers et al. [4]. In this work, Poppe plots [5] were used to determine the first dimension (^{1}D) experimental conditions, with the sampling time (t_s) fixed equal to the ^{1}D peak standard deviation (a more commonly accepted norm for t_s is 1.5-3$^1\sigma$ [6]). The same Poppe approach was used to determine the second dimension (^{2}D) conditions. Injection band broadening and dilution were minimised by selecting the appropriate column diameters.

An alternative approach is to perform step-wise method optimisation, which offers the benefit of relative simplicity [7]. For example, Guiochon and co-workers [8] used a fixed ^{1}D separation

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in combination with accepted undersampling criteria and linear solvent strength (LSS) calculations to establish performance as a function of ^{2}D gradient time. Carr and co-workers [9] reported two approaches for the optimisation of peak capacity in LC × LC. In the first two-step procedure, ^{1}D peak capacity at a selected gradient time was initially maximised, followed by optimisation of the ^{2}D separation by varying t_s and considering both the effect of ^{1}D undersampling and ^{2}D gradient time on the effective two-dimensional peak capacity ($n'_{c,2D}$). For one-step optimisation, the maximum $n'_{c,2D}$ was determined by simultaneously varying four ^{1}D parameters as well as t_s using the Solver function in Excel.

In 2010, Vivó-Truyols et al. [10] reported the most complete kinetic optimisation protocol for on-line LC × LC yet, where Pareto-optimisation (simultaneous optimisation of more than one objective function) was used to determine optimal conditions in terms of total analysis time, peak capacity and dilution. The important chromatographic parameters (t_s, particle sizes, column dimensions and flow rates) were allowed to vary either continuously or in discreet intervals. The effect of undersampling (treated as detection band broadening) and injection band broadening were also taken into account. Subsequently, Heinisch and co-workers [11] used a step-wise Pareto approach to optimise the RP-LC × RP-LC separation of peptides. The effects of undersampling, injection band broadening, dwell volume and re-equilibration time in the ^{2}D were taken into account, as was the option of splitting the ^{1}D eluent to reduce fraction volumes. The analysis time, sampling rate and ^{2}D internal diameter (^2d_c) were fixed prior to optimisation and the ^{2}D separation was operated at maximum pressure. This approach was subsequently modified, notably by including the sampling rate as variable, to determine the crossover time where RP-LC × RP-LC outperforms fully optimised 1D RP-LC separation of peptides [12].

Step-wise method optimisation protocols inherently suffer from the limitation that important parameters such as column diameters and particle sizes, column lengths and sampling times are pre-selected, thereby limiting their scope – although these methods are useful for determining the optimal conditions for a given column set, for example, they do not provide information on the optimal column set and conditions to operate these.

More complete optimisation protocols such as those based on Pareto-optimisation are also far from universal as a consequence of the impact of the chosen fundamental relationships (and their accuracy) used to model the effect of particular experimental variables, as well as the sequence in which they are optimised. For example, the use of freely (as opposed to step-wise) variable column dimensions provides practically irrelevant results [4], whereas fixed column dimensions clearly limit the generic application of multi-parameter optimisation approaches [11]. Similarly, fixed sampling rates potentially exclude optimal configurations [4,11]. Another common specification is that the ^{2}D column should be operated at maximum pressure; although this makes sense from a kinetic performance point of view, this might exclude optimal LC × LC configurations where lower dilution is gained by sacrificing kinetic performance [4,10–12]. A further aspect worthy of note is that experimental verification of the accuracy of predicted performance is still largely lacking. Sarrut et al. reported significant (50–70%) overestimation of actual performance [11], whereas subsequent modification of the optimisation protocol and correction for ^{2}D extra-column variance drastically improved the accuracy of the method [12].

To take full advantage of the increased separation space available in LC × LC, different separation mechanisms must be used in each dimension [1]. Various combinations of size exclusion chromatography (SEC), ion exchange, normal phase LC (NP-LC), hydrophilic interaction chromatography (HILIC) and RP-LC have been used with success in LC × LC, depending on the analytes under investigation. Due to the high degree of orthogonality associated with HILIC and RP-LC separations [13], their comprehensive combination has received particular attention, and application, in the fields of peptide, lipid, phenolic and polymer analyses [14]. The coupling of HILIC and RP-LC separations however presents unique challenges associated with the high elution strength of HILIC mobile phases in RP-LC, which can potentially severely compromise method performance, and must therefore be considered during method optimisation. In a study comparing the performance of RP-LC × RP-LC and RP-LC×HILIC for peptide separations, it was shown that although surface coverage of the RP-LC×HILIC system was superior, the effective peak capacity was lower than RP-LC × RP-LC due to severe band broadening when injecting undiluted ^{1}D effluent in the HILIC dimension [15]. Kalili et al. [16,17] reported a practical approach to method optimisation in HILIC × RP-LC, where injection band broadening was avoided by pre-determining the maximum injection volume ($^2V_{inj,max}$) on a selected ^{2}D column, and splitting the ^{1}D effluent accordingly. Since the column sizes were preselected, this approach can be regarded as a step-wise method development scheme rather than a true optimisation procedure.

It should be pointed out that most kinetic optimisation protocols for LC × LC rely on the pre-selection of thermodynamic parameters (i.e. stationary phases, mobile phases and temperatures) based on optimised 1D methods. The assumption is therefore that the separation modes used in each dimension are selected based on selectivity (orthogonality) considerations for the sample of interest prior to kinetic optimisation. In contrast, optimisation of mobile phase gradients to increase the resolution and orthogonality of LC × LC separations has recently been addressed by Pirok et al. [18].

In this study we report a comprehensive kinetic optimisation protocol for on-line HILIC × RP-LC separations. Mobile and stationary phases were pre-selected based on previous work [16,17,19,20]. The methodology applies Pareto-optimisation, with the following key features: (1) only practically feasible (user defined) column dimensions and flow rates based on the available instrumentation are considered, (2) cases where both the first and second dimension columns are operated below the maximum pressure are included, (3) column void times and system volumes are taken into account, (4) the options of splitting and/or diluting the ^{1}D eluent are included and (5) due to the lack of reliable injection models, restrictions were added to ensure negligible injection band broadening in the ^{2}D. The validity of the approach is demonstrated through experimental verification of the performance for the HILIC × RP-LC separation of procyanidins, an important class of condensed tannins of the flavonoid family, in a cocoa extract.

2. Theory

In the following, the equations describing the relationships between experimental variables used in the optimisation protocol are briefly outlined.

2.1. Analysis time

In on-line LC × LC, the total analysis time ($^{2D}t_{total}$) is approximately equal to the ^{1}D gradient time (^1t_g). Since both the void volume ($^1V_0$), calculated from the column dimensions, and dwell volume (^1V_D), a system property, are in volume units, the gradient time in the ^{1}D (^1t_g) can be calculated for any first dimension flow rate (^1F):

$$^1t_g = {}^{2D}t_{total} - \frac{(^1V_D + {}^1V_0)}{^1F} \quad (1)$$

In the same manner, the ^{2}D gradient time (^2t_g) can be determined from the sampling time (t_s, which in on-line LC × LC is equal to the ^{2}D cycle time, ^2t_c) if the flow rate (^2F), void volume ($^2V_0$),

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