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A generic approach to post-column refocusing in liquid chromatography

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ABSTRACT

To increase detection sensitivity in liquid chromatography, a generic post-column refocusing strategy has been developed to enrich (target) analytes prior to detection. In this strategy, after separation on the analytical column, the analytes are led to a trap column preferably containing a stationary phase with strong retentive properties (*e.g.* silica C_{30}). They are then eluted using a strong solvent in a backward-elution mode. A first key element of the proposed strategy is that the trapping time should be at least equal to the time the front of the remobilization solvent needs to cover the entire length of the trap column, divided by the ratio of the flow rates used for trapping and remobilization. This condition is independent of the retention properties of the analytes in the trapping and remobilization solvent. Another essential element is the addition of a third solvent (isopropanol in the present case) to the remobilization solvent to overcome viscous-fingering effects caused by the viscosity difference between the trap and the remobilization solvents. The potential of the proposed post-column refocusing strategy is demonstrated for an isocratic separation of KI (t_0 marker), an antibiotic (sulfamethazine), and acetophenone as a case study. Using optimized remobilization conditions a maximum signal-enhancement factor of 8 was achieved. Higher enhancement factors using a remobilization solvent with slightly higher elution strength were prohibited by disturbances of the UV background signal.

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1. Introduction

High-performance liquid chromatography (HPLC) has emerged as one of the most important techniques for the analysis of non-volatile (contaminant) samples and is successfully applied in many application areas. However, the fundamental nature of the separation process is such that analytes in a sample mixture are diluted when components are distributed between mobile and stationary phases. Dilution is a problem for impurity profiling, in which the aim is the detection, identification/structure elucidation, and quantification of sub-ppm components and residuals in a wide variety of matrices [1]. Advances in column technology and the development of UHPLC instrumentation have led to better separation efficiency and hence increased detection sensitivity because analytes elute in narrower highly concentrated zones [2,3]. Furthermore optimization of the design of detector flow cells and advances in mass spectrometry interfacing *via* electrospray ionization (ESI), has resulted in considerable improvements in detection sensitivity [4–6]. However, even when HPLC is hyphenated to sensitive detectors such as mass spectrometers (MS), the detection limits are not always low enough to detect at the required trace levels set by authorities. For example, the detection and accurate analysis of trace-levels of antibiotics and their metabolites in agricultural and food products is a major bottleneck and several classes are even undetectable using current LC–MS methods [7]. The presence of sub-therapeutic doses in food products increases the prevalence of antibiotic-resistant bacteria [8]. In addition, concerns have been raised regarding the carcinogenicity of the residues [9]. When considering small-molecule method development in two-dimensional liquid chromatography (LC × LC), dilution is an even larger bottleneck since the dilution factors in the two consecutive chromatographic processes are multiplicative when analyte focusing between the two developments is not possible [10,11].

On-column preconcentration procedures in HPLC have been described by several authors as a means to enhance detection limits [12,13]. However, when the sample volume is increased, the injected volume will cover a more significant fraction of the column, decreasing column performance, which in turn severely compromises sensitivity of the analytical method. Hence, a trade-off needs to be made between detection limits and chromatographic resolution. A solid-phase-extraction (SPE) column is often applied prior to the

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analytical column using a 6-port switching valve, allowing large-volume injections. The combination of stationary phases and mobile phase is selected in such a way that the retention of the target analyte(s) is excessively large in the trapping solvent on the SPE column and moderate in the mobile phase applied for the analytical separation [14]. In addition, the possibility to apply low trap-column temperatures has been explored to increase retention factors [15]. However, chromatographic dilution will still decrease detection sensitivity.

An alternative approach is to apply trapping and remobilization of target analytes post-column prior to detection. This approach has been widely employed in HPLC-NMR workflows to preconcentrate target analytes and, in a second step, realize a solvent switch towards deuterated solvents [16,17]. Despite its success, this approach has not been widely adopted in other hyphenated strategies or with different detectors such as UV or MS.

In the present study, the possibilities and limitations of the post-column refocusing are investigated and its feasibility is demonstrated for the isocratic reversed-phase separation of an artificial mixture containing the t_0 marker, an antibiotic (sulfamethazine), and acetophenone, as a case study. The compounds and elution conditions were selected to induce a large retention time difference allowing to investigate the effect of the retention-factor ratio experienced on the analytical column and trap column (applying different remobilization solvents) on signal enhancement. Hence, the test mixture does therefore not represent a real application example. In addition, the influence of the trapping and remobilization time, and the optimal choice of solvent combinations on the achievable concentration factors is established.

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile (ACN, HPLC supra-gradient quality) and formic acid (FA, 99%) was purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Potassium iodide (KI), sulfamethazine, acetophenone, and isopropyl alcohol (IPA) were obtained from Sigma–Aldrich (Bornem, Belgium). Deionized HPLC-grade water (≤0.055 μS) was produced in-house using a Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Chromatography

Chromatographic experiments were performed using a customized HPLC instrument (Agilent Technologies, Waldbronn, Germany) composed of a 1100 series quaternary pump (for the analytical separation), a 1200 series autosampler, a 1100 series thermostated column compartment equipped with a 2 position 6-port valve, a 1200 series binary pump (supply for remobilization solvent), a 1200 series thermostated column compartment equipped with 6 μL solvent preheater (for applying a constant temperature over the remobilization solvent), and a 1200 series diode array detector equipped with a micro high-pressure detector cell (1.7 μL cell volume). Data acquisition was performed using Agilent Chemstation software (Rev. B.03.02 [341]). A 2.1 mm i.d. × 150 mm Hypersil Gold column packed with 1.9 μm silica C_4 particles (Thermo Fisher Scientific, Runcorn, UK) was used for the analytical separation. The trap column was a 2.1 mm i.d. × 150 mm Accucore column packed with 2.6 μm silica C_{30} core-shell particles (Thermo Fisher Scientific).

The analytical separation was conducted in isocratic mode applying a mobile-phase composition of 90:10% (v/v) water:ACN containing 0.1% (v/v) formic acid at a flow rate of 0.11 mL/min (optimum mobile-phase velocity). The analytical separation was conducted at 20 °C. The trap column was connected to the analytical column *via* a 6-port valve. Prior to trapping the trap column was always equilibrated with 90:10% (v/v) water:ACN containing 0.1% (v/v) formic acid. After trapping a target component on the trap column, the remobilization solvent composed of ACN, water, and isopropyl alcohol in different ratios containing 0.1% (v/v) formic acid, was applied in back-flush mode to transfer the target component to the detector using a flow rate of 0.11 mL/min. The temperature of the remobilization solvent was maintained at 20 °C. UV detection was performed at a wavelength of 200 nm (0.2 s response time).

3. Results and discussion

3.1. Post-column refocusing: instrument set-up and minimal trapping time

Fig. 1 shows the instrument set-up used to investigate the possibilities and limitations of signal enhancement that can be obtained using the proposed strategy, applying a conventional normal-bore analytical column and trap column, respectively. Target components eluting from the analytical column with initial peak width were directed to the trap column (heart-cut LC) *via* a six-port valve (Fig. 1A). When the trap column contains a stationary phase with stronger retentive properties (*e.g.* silica C_{30}) than that of the analytical column (*e.g.* silica C_4), an on-column focusing effect can be obtained (Fig. 1B). To fully benefit from this on-column concentration enhancement at the detector, a second step is needed wherein the trapped peak is remobilized using a strong remobilization solvent that can overtake the peak during its elution from the trap column. In the present study, the remobilization is performed in back-flush mode to minimize the elution path length, hence decreasing band broadening as much as possible (Fig. 1C).

The fact that a peak-sharpening effect can only be expected when the front of the remobilization solvent is able to catch up with the backward eluting trapped band during the remobilization step is demonstrated in Section 3.4. As demonstrated below, this condition implies that a minimal trapping time needs to be respected. To understand this, the travel-distance parameters δ_{trap} and $\delta_{overtake}$ defined in Fig. 1D can be used, with δ_{trap} the distance covered by the band during the trapping step:

$$\delta_{trap} = \frac{u_{0,trap} \cdot t_{trap}}{(1 + k'_{trap})} \quad (1)$$

$\delta_{overtake}$ is the distance covered by the band during the elution step at the moment where the gravity centre of the species band is overtaken by the front of the remobilization solvent. The condition needed for the remobilization solvent to overtake the backward eluting band can then be written as:

$$\delta_{trap} \geq \delta_{overtake} \quad (2)$$

The distance $\delta_{overtake}$ can be calculated by expressing that the time needed by the front of the remobilization solvent to catch up with the centre of the backward-eluting species band (travel speed = $u_{0,remob}$; travel distance = $(L_{trap} - \delta_{trap}) + \delta_{overtake}$) is equal to the time needed by the species band to cover the distance $\delta_{overtake}$. In this case, the travel speed is $u_{0,remob}/(1 + k'_{trap})$ because during this displacement the band still moves in the trapping solvent. Expressing this mathematically, this leads to:

$$L_{trap} - \delta_{trap} u_{0,remob} + \frac{\delta_{overtake}}{u_{0,remob}} = \frac{\delta_{overtake}}{u_{0,remob}} \cdot (1 + k'_{trap}) \Leftrightarrow \delta_{overtake} = \frac{L_{trap} - \delta_{trap}}{k'_{trap}} \quad (3)$$

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