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Switching solvent and enhancing analyte concentrations in small effluent fractions using in-column focusing[☆]

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ABSTRACT

A novel approach to achieve solvent switching and focusing of sub-column-volume analyte fractions in liquid chromatography is presented. By altering the temperature between loading and elution in back-flush mode, solvent transfer of analytes and focusing occurs, provided that the analytes exhibit temperature dependent retention on a given trap column. When retention on the trap decreases with increasing temperature, which is almost always the case, the loading of the trap-column takes place at a higher temperature than the elution. This principle is demonstrated using three small aromatic molecules (toluene, p-xylene and benzophenone) on a capillary monolithic column. On this column, the analytes show a traditional van't Hoff dependence on temperature with enthalpy effects of, -15 , -16 and -18 kJ mol⁻¹, respectively, for a mobile phase of 25% acetonitrile in water. The column was loaded at 110 °C, cooled in an ice bath and eluted in back-flush mode at 0 °C. When operated in this way, the analytes are transferred from the loading solvent to the elution solvent, achieving solvent switching. Substantial focusing can also be obtained if the desorption solvent is stronger than the loading solvent.

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1. Introduction

Comprehensive two-dimensional liquid chromatography (LC × LC) is the separation approach of choice for detailed analysis of complex, non-volatile mixtures. This is because LC × LC provides higher separation power (i.e. peak capacities) than one-dimensional LC (1D-LC) within a reasonable time [1] and because LC × LC provides additional selectivity. When the sample dimensions match the separation dimensions [2] LC × LC also yields structured, readily interpretable chromatograms [3]. A standard interface between the first (¹D) and second (²D) separation dimensions consists of a switching valve equipped with two collection loops. Both dimensions are run continuously, as the two loops are alternately switched between collecting a ¹D fraction and injecting it into the ²D [4,5]. Following common nomenclature from comprehensive two-dimensional gas chromatography (GC × GC) [6,7] this process is called modulation. In LC × LC operational drawbacks that can be attributed to the collecting interface often hamper full

realization of the method's resolving power. Factors commonly mentioned in literature as contributors to modulation-induced decrease in efficiency of LC × LC analyses are (1) asymmetry of collection loops [5], (2) lengthy modulation cycles [8], (3) viscosity mismatch [9,10], (4) solvent immiscibility or incompatibility [11], (5) ¹D eluent being too weak or too strong an injection solvent in ²D and (6) large ²D injection volumes [12–14]. Two different (“asymmetrical”) collection loops can cause significant differences in consecutive (odd and even) ²D runs [5]. Different loops may also lead to differences in band broadening and peak shapes. A poorly connected or partially blocked loop may lead to peak distortion in the odd or even series of chromatograms. Long modulation cycles can compromise the separation obtained in the first dimension due to under-sampling and can result in relatively large ²D injection volumes, which may add significantly to the second-dimension band broadening [15,16]. Incompatibility of the ¹D effluent and the ²D mobile phase can give rise to several problems. Viscosity mismatch of eluents can cause viscous fingering, which may distort the solute band profiles [17]. The ¹D elution solvent can be a strong injection solvent for the ²D, which can cause fronting, tailing, band broadening and other peak distortions [18]. Peak splitting [13] and breakthrough of analyte peaks [12] have been reported as dramatic consequences of solvent incompatibility. Finally, the first-dimension solvent may be incompatible with the detection method envisaged after the ²D separation [15]. Overall, these

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effects combine to diminish the potential of LC $\times$ LC analysis [15]. To combat these disadvantages and achieve the full resolution potential of two orthogonal separation mechanisms, an ideal modulation interface should realize a change of solvent and focus the 1D elution band, while re-injecting in a relative short time without any discrimination between the sample components [19].

Modulation in comprehensive two-dimensional gas chromatography (GC $\times$ GC) has been studied in depth and is routinely employed in a (near-) universal and robust way [20]. In GC $\times$ GC the dominant modulation principle is based on effects of temperature on retention, trapping the compounds from the 1D by rapid local cooling and re-injecting into the 2D by rapid heating. Analogously, in LC $\times$ LC, modulation based on thermal effects on retention has not yet been demonstrated. However, temperature effects have been used for trap and release purposes in LC [21–25]. In LC $\times$ LC diluting make-up flows between separation dimensions are more commonly employed to mitigate the aforementioned drawbacks at the expense of higher complexity and possibly reduced sensitivity [26,27]. The most-promising LC $\times$ LC modulation interface consists of a trapping column between the dimensions to trap the analytes, focus them and change solvents [28]. The use of such a packed-loop interface requires loading of multiple trap-column volumes (possibly resulting in loss of separation achieved by the first dimension), expert know-how, time-consuming optimization, high retention factors and miniaturization [29–31]. Packed-loops have, however, proven their value in LC $\times$ LC modulation [32–39]. Other modulation principles, such as eluent-evaporation approaches, have been explored, but these have found little acceptance, due to limited applicability, high complexity or lengthy time-cycles [40,41].

To best carry out solvent switching and focusing desired for LC $\times$ LC modulation, a miniaturized packed loop interface should be operated in back-flush elution mode. Back-flush elution has been shown to have benefits over the more traditional forward flush elution mode both in cycle time and focusing [30,31]. In the context of LC $\times$ LC modulation, miniaturized packed loops require high retention factors if multiple column volumes of 1D effluent are to be loaded. This may lead to effective concentration and solvent switching, but modulation times may be longer, low-retained analytes may be lost and contaminants from the eluent will be focussed together with the analytes. The system may also be difficult to generalize and optimize in case the effluent that needs to be modulated arises from a (first-dimension) gradient-elution separation. Loading only a single trap-column volume or less cannot result in analyte solvent transfer or focusing when performed in isothermal back-flush mode. The analytes would elute in the loading solvent and never transcend the loading-elution solvent boundary. A chromatographic column (or trap) is completely filled (*i.e.* one trap-column volume) with a solution containing (parts of) one or more analyte bands (*e.g.* the effluent from a previous separation stage). This is illustrated schematically in Fig. 1A where isothermal loading (a–d) and back-flush elution (d–g) take place without any solvent switching or focusing. Therefore, there is a need to increase the retention of analytes whilst on column, still in the loading solvent, if solvent change of single or sub-trap-column volume is desired. An effective way to achieve this is by varying the temperature. The current trend of using temperature to increase trapping efficiency on trap-columns relies on cold-traps that are heated up at the time of elution. For sub-column volumes, however, this approach fails. In

Fig. 1. Loading and elution schemes. Schematic representation of loading (a–d) and back-flush elution (d–g) of an analyte plug composed of three analytes ($\square$, $\circ$, $\triangle$) with different retention. The analyte plug is injected (a) and loaded with one trap volume of mobile phase (the loading solvent). Fig. A illustrates a constant-temperature process. In figures B, C and D a temperature change takes place (d). A high column temperature is illustrated with a red backdrop (H) and a low temperature is shown in blue (C). In D, X marks the imaginary position of analytes, had they not been swept up. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 1B, cooling the column at the time of loading is depicted (a–d). After one trap-column volume has been loaded, the temperature is increased (d). Upon elution in back flush-mode the analytes do not change solvents due to reduced retention at the higher temperature (d–g). This method is more commonly applied for larger effluent volumes (multiple volume of the trapping column) per modulation. In this paper we present a novel modulator setup that allows both focusing and solvent switching. The loading step is performed at elevated temperature (Fig. 1C, a–d). Next, the trapping column is cooled and eluted in a back-flush manner (Fig. 1C, d–g). This induces lower analyte retention entering the column than when eluted. When operated in this way, the analytes are transferred from the loading solvent (dotted yellow area) into the elution solvent (clear yellow, Fig. 1C), achieving solvent switching as discussed in Section 3.2. If the elution solvent is of a high enough elution strength, it will sweep up the analytes when they transcend the solvent boundary (Fig. 1D) and in-column focusing (ICF) will occur as discussed in Section 3.3. In principle, the presented method is suitable for all molecules that exhibit a (significant) decrease in retention upon increasing the temperature (on the modulator column), irrespective of the loading solvent. ICF may also be applied in situations in which retention increases with increasing temperature. Such situations are much less common, but it may occur in some cases, for example when the effluent that requires modulation is a supercritical fluid. In the present study thermal effects on retention are investigated for small-molecules on a reversed phase monolithic column and the combinations of retention-affecting parameters (loading conditions, temperature changes and elution conditions) are explored in pursuit of the “ideal modulator”.

2. Experimental

2.1. Materials, analytes and solvents, monolithic-column preparation

Ethylene dimethacrylate (EDMA, 98%), butyl methacrylate (BuMA, 99%), 3-(trimethoxysilyl) propyl methacrylate (γ -MPS, 98%), 2,2'-azobisisobutyronitrile (AIBN, 98%), 1-decanol (99%), toluene (99.9%) and aluminium oxide were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Methanol (MeOH), acetonitrile (ACN), acetone, p-xylene, benzophenone and tetrahydrofuran (THF, >99.8%) were purchased from Biosolve (Valkenswaard, The Netherlands). Thiourea (>99%) was purchased from BDH Chemicals (Poole, England). Sodium hydroxide (NaOH) was purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) (37%) was obtained from Acros (Geel, Belgium). Milli-Q water (18.2 M Ω cm) was produced by a Sartorius Arium 611UV Ultrapure Water System (Goettingen, Germany). Fused-silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ, USA) (200 μ m i.d., 360 μ m o.d. for the monolithic column and 50 μ m i.d., 360 μ m o.d. for the connections). Capillary tubing was connected using IDEX20 Health & Science P-772 Micro tight unions (Mondfeld, Germany). A 150 mm long butyl methacrylate co-ethylene dimethacrylate (BuMA-EDMA) monolithic column was made using a polymerization time of 45 min following the procedure described before [42]. An analyte mixture was prepared to contain final concentrations of 0.6 mg/mL thiourea, 12.1 mg/mL toluene, 10.5 mg/mL p-xylene, and 26.3 mg/mL benzophenone in 99:01 ACN:water (by volume).

2.2. Instrumentation

The instrumental set-up used is schematically illustrated in Fig. 2. Loading of the trapping column was performed with an Agilent Technologies 1100 Series pump at 10 μ L/min, equipped with a

Fig. 2. Experimental setup. A schematic depiction of the instrumental setup used. When Valve 1 is in position A (indicated by black lines), Pump 1 loads the column. When valve 1 is switched to position B (denoted by gray lines) the column is brought offline with pump 1 and online with the detector D. After a period of cooling, valve 2 is switched to position B and the column is eluted in a back-flush manner.

G1379A degasser (Agilent Technologies, Waldbronn, Germany). A Shimadzu CBM-20A controller recorded the UV trace from a Linear UVIS200 detector (Shimadzu, 'sHertogenbosch, The Netherlands). Valve 1 was a VICI EHMA electronically actuated valve, while valve 2 was a Cheminert C2-2000D air-actuated valve (Valco VICI International, Schenkon, Switzerland). 20 nL of sample were injected using a Cheminert C4-0344-02 manual injector. Back-flushing of the column was performed using a Shimadzu LC-10A Dvp pump (pump P2) at 10 μ L/min. The trapping column was heated using a Waters column-heating module (Milford, MA, US) and cooled by immersion in an ice bath.

2.3. Experimental procedure

Three sets of experiments were performed. First, the effects of temperature on retention were established for the given test analytes, monolithic column, and loading and elution solvents (Section 3.1). Next, the possibility of solvent switching was explored (Section 3.2) and lastly the focusing capabilities of the setup were demonstrated (Section 3.3). For measuring the effects of temperature on retention, valves 1 and 2 were kept in position A (denoted by a black line, Fig. 2) in a traditional pump-injector-column-detector arrangement for the duration of the run. The column was placed in a column heater at 110 °C or in an ice bath for cooling at 0 °C. Pump 1 was delivering 25% ACN in water at 10 μ L/min.

For exploring solvent switching, the 25% ACN mobile phase of pump 1 was spiked to contain 0.033 mg/mL thiourea. At $t = 0$ s the column was equilibrated at 110 °C and the analytes were injected. At $t = 20$ s, valve 1 was switched to position B (denoted by a gray line Fig. 2) and the column was immersed in the ice bath. At $t = 630$ s, valve 2 was switched to position B to elute the analytes in a back-flush manner with 25% ACN (Section 3.2). The same experimental procedure was followed to explore in-column focusing (Section 3.3), except that the back-flush solvent was 40% ACN and there were no additives added to the mobile phase.

3. Results and discussion

3.1. Thermal effects on retention

Temperature effects on retention are well explored, but their magnitude depends on the mobile and stationary phase and on the analytes [43]. To measure the effect in the chromatographic system used in this work, measurements with the monolithic column heated to 110 °C (drawn line, Fig. 3) were compared to measurements at 0 °C (dashed line). At 110 °C toluene elutes at about 3 min (retention factor of about 2), while p-xylene and benzophenone co-elute around 4 min with retention factors of about 3. At 0 °C, the analytes are well separated with retention factors of 10, 20 and 27, respectively. Assuming linear (van't Hoff) behaviour across the entire range, this corresponds to ΔH values of about –15, –16 and –18 kJ mol^{–1}. These values agree with literature data of Ueki et al. who studied the retention behaviour of alkyl benzenes on a monolithic column [44] created using a monomer featuring an octadecyl

Fig. 3. Thermal effect on retention. Drawn line shows the retention of three analytes at 110 °C. Dashed line shows retention at 0 °C. Separations were performed on a BUMA-EDMA monolithic column with 25% ACN in the mobile phase. Flow rate, 10 μ L/min and UV detection at 254 nm. The t_0 marker (thiourea) is indicated with an *.

chain. They obtained a ΔH value for toluene of -16 kJ mol^{-1} . Our values are also comparable to those reported by Verstraeten et al. [21], although these authors used quite different analytes and stationary phases.

3.2. Solvent switching

Solvent switching of analytes is an integral part of many analytical methods. In LC $\times$ LC modulation, solvent switching aims to transfer analytes from the effluent of the first dimension (1^D) to a more compatible injection solvent for the second dimension (2^D). To investigate the solvent-switching capabilities of the instrumental setup, a tracer was added to the loading solvent. This would

make the solvent boundary between the loading and elution solvents observable with a UV-detector. This tracer in the loading solvent shows up as a “box” on the recorded UV-trace (dotted line Fig. 4) and this represents the loaded column volume of solvent contained within the trap-column. To demonstrate the solvent switching capability of the method, a comparison between constant temperature (Fig. 1A) and the hot loading, cold elution (Fig. 1C) mechanisms is presented in Fig. 4. When constant temperature is employed (dashed line) all analytes co-elute with the unretained signal, within the loading solvent (peak marked with *), presenting no benefits. When the hot loading, cold elution method is employed (drawn line), the analytes transcend the solvent boundary, from the loading solvent into the elution solvent. A part from an unretained

Fig. 4. Solvent switching. Example of 254-nmUV trace obtained from a constant-temperature experiment (dashed line, Fig. 1A) compared to a hot loading, cold elution approach (drawn line, Fig. 1C). Dotted line shows a blank injection. The tracer from the loading solvent shows up as a “box” from about 11 min to about 13.5 min. Marked are system peak (*) and t_0 peak (*).

Fig. 5. In-column focusing. Comparison of 254-nm UV traces obtained from hot loading, cold elution approach with 25% ACN (drawn line) and 40% ACN (dashed trace) as (back-flush) elution solvents. The 40% ACN solvent sweeps up the trapped analytes, concentrating them in one band. Marked are system peak (*) and t_0 peak (*).

signal on the right-hand side of the “box”, there is also a peak at the front end of the “box”. This system peak (marked with ★) arises from restarting the solvent flow through the detector after the pause for cooling.

3.3. In-column focusing

To investigate the focusing achievable by the instrumental setup, a comparison between back-flush elution with 25% ACN and with 40% ACN was made (Fig. 5). Elution with 25% ACN (drawn line in Fig. 5) yields broad peaks with low intensity while elution with 40% ACN (dashed line) sweeps up the analytes and focuses them in a much more intense peak. The extent of focusing that can be achieved by this method depends on a number of factors. Substantial focussing is demonstrated in Fig. 5.

4. Conclusions

In this work a novel trapping method with solvent switching and focusing capability has been presented. This mechanism is in principle applicable to all analytes that show a significant and monotonic variation of retention with temperature on a suitable trap-column. It can therefore be considered a near-universal modulation principle. The working principle has been demonstrated for three low-molecular-weight model compounds. The sample solvent was switched and the elution plug was focused using in-column focusing. The prototype set-up used in this work is slow compared to the requirements needed for modulation in LC × LC. The modulation in LC × LC has to be fast and robust, because hundreds of modulations are commonly performed during each analysis. Future work will be aimed at reducing the time needed for heating and cooling and at enhancing the robustness of the setup via resistive heating and active cooling. However, the solvent switching capabilities and focusing potential of in-column focusing have been clearly demonstrated.

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