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Journal:	*Analytical Chemistry*
Manuscript ID	ac-2017-04914f.R1
Manuscript Type:	Technical Note
Date Submitted by the Author:	09-Mar-2018
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Enhancing the possibilities of comprehensive two-dimensional liquid chromatography through hyphenation of purely aqueous temperature-responsive and reversed-phase liquid chromatography

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ABSTRACT: Comprehensive two-dimensional liquid chromatography (LC×LC) allows for substantial gains in theoretical peak capacity in the field of liquid chromatography. However, in practice theoretical performance is rarely achieved due to a combination of undersampling, orthogonality and refocusing issues prevalent in many LC×LC applications. This is intricately linked to the column dimensions, flow rates and mobile phase compositions used, where in many cases incompatible or strong solvents are introduced in the second-dimension (^{2}D) column, leading to peak broadening and the need for more complex interfacing approaches. In this contribution, the combination of temperature-responsive (TR) and reversed-phase (RP) LC is demonstrated, which, due to the purely aqueous mobile phase used in TRLC, allows for complete and more generic refocusing of organic solutes prior to the second-dimension RP separation using a conventional 10-port valve interface. Thus far this was only possible when combining other purely aqueous modes such as ion exchange or gel filtration chromatography with RPLC, techniques which are limited to the analysis of charged or high MW solutes, respectively. This novel TRLC×RPLC combination relaxes undersampling constraints and complete refocusing, and therefore offers novel possibilities in the field of LC×LC including temperature modulation. The concept is illustrated through the TRLC×RPLC analysis of mixtures of neutral organic solutes.

Introduction

Two-dimensional liquid chromatography

Over the last decades comprehensive two-dimensional liquid chromatography (LC×LC) has increasingly been used to provide additional resolving power for the satisfactory separation of very complex samples, where the state of the art one-dimensional and heart-cutting approaches often fall short.[1–5] The improved separation performance of LC×LC is a result of the fractional transfer of the first-dimension (^{1}D) eluent to a second complementary separation, allowing ideally for a theoretical peak capacity equal to the product of the peak capacities of both modes. However, robust practical implementation of this technique is not without its own unique set of obstacles, which often detrimentally affect the figures of merit of LC×LC in practice.[6] The first of these involves under-sampling, which refers to the potential loss of ^{1}D resolution when the sampling frequency is insufficient to retain separation in the ^{1}D. Murphy et al. showed that ideally each ^{1}D peak should be sampled 3 to 4 time in order to minimize the loss of ^{1}D resolution.[7] This implies that very fast ^{2}D analyses are required in online LC×LC. Nowadays, fast (U)HPLC gradient analyses allow minimization of the under-sampling constraint.[8]

Secondly, unavoidable correlations in selectivity between separation dimensions limit optimal use of the separation space in LC×LC.[9] The consequent loss of separation space due to this lack of orthogonality has a detrimental effect on the achievable peak capacity compared to the theoretical maximum.[10] Few LC×LC combinations fully comply with the requirement of orthogonality.

The most challenging aspect of LC×LC, however, is the modulation process, which deals with the complex issue of transferring fractions between the ^{1}D and ^{2}D. When loading a fraction onto the ^{2}D column, the inherent volume of the fraction, coupled with inadequate refocusing of the sample on the ^{2}D column, can lead to significant injection band broadening and therefore loss in ^{2}D peak capacity.[11,12] This effect constrains the design of LC×LC setups to micro-bore ^{1}D columns, which negatively impacts sample capacity and sensitivity. Furthermore, this also complicates the development of robust LC×LC-MS platforms, as most contemporary methods require largely discrepant flow rates in ^{1}D (~1-200μL/min) and ^{2}D (~1-5 mL/min). Modulation challenges occur to varying degrees in most column combinations used in LC×LC, and indeed can be so problematic that particular combinations are deemed unusable. Only a few combinations are exempt from these modulation constraints as they offer inherent on-column refocusing, for example ion exchange x RP chromatography or aqueous size exclusion x RP chromatography.[13,14]

To overcome these issues, several alternative modulation interfaces have been developed to promote refocusing of the

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analytes at the start of the ^{2}D column,[15] including: [1] dilution of the ^{1}D eluent with a weak solvent, making it more compatible with the ^{2}D stationary phase[16–18], [2] splitting the ^{1}D flow,[19,20] [3] active modulation, in which trapping columns are used to refocus the analytes to remove of the bulk of the ^{1}D eluent, [16,21,22] [4] temperature modulation, which uses cold temperatures to trap the analytes,[23–26] [5] partial evaporation, which removes part of the fraction volume by selective evaporation of the mobile phase,[27,28] and [6] solvent switching, whereby the analytes are transferred from the ^{1}D mobile phase into a more compatible mobile phase.[29] Each of these approaches provide an adequate solution to particular combinations of separation modes, yet all of them come with certain penalties, including potential loss of ^{2}D separation time or extreme instrumental complexity. Clearly, there is a need for improved LC×LC methodologies that circumvent some of the constraints associated with the modulation process.

Temperature-responsive chromatography

The use of polymer-derived stationary phases in liquid chromatography, either as a replacement for or as a hybrid silica-based stationary phase, has been expanding in the last decades. An interesting discovery in this field has been the development of temperature-responsive (TR) stationary phases, in which a TR polymer is used to achieve separation.[30–32] This type of polymer is classified as an "intelligent or smart polymeric material", as it exhibits a sharp change in physical properties upon small changes in its environment. In the case of TR polymers, they possess a unique characteristic that causes them to change their water solubility based on the temperature. This means that, for every polymer/water composition, a specific temperature, called the cloud point, exists at which the polarity of the polymer will shift from water-soluble to water-insoluble. The lowest of these temperatures over all polymer/water compositions is called the Lower Critical Solution Temperature (LCST), below which the polymer is water-soluble in all compositions. This phenomenon is caused by intermolecular interactions, which lead to a decrease in polarity when increasing the temperature.[33] It is this property that is exploited, as the use of a temperature-responsive polymer-based column allows the stationary phase polarity to be controlled through control of the column temperature, which offers the option of performing LC separations in purely aqueous mobile phases (see Figure 1) as alternative to RPLC.

Poly(N-isopropylacrylamide) (PNIPAAm) is the polymer that has been explored most often for use in temperature-responsive liquid chromatography (TRLC).[34] This polyacrylamide is an ideal candidate for use in TRLC as it is very stable and versatile, allowing for several coupling chemistries to be applied to attach the polymer to the silica support. Furthermore, the typical cloud point of the polymer (32ºC) is situated in a convenient temperature range where the viscosity of water is not excessive, the hydrothermal stability of the (silica) supporting materials is not exceeded and where analyte degradation is also improbable. Alongside columns based on homo-PNIPAAm, several co-polymers including NIPAAm have been tested as well, as they lead to a modification of the LCST and induce slightly different retention properties.[35–42] Other polymers that have successfully been used in TR columns include polyoxazoline (LCST controllable) and polyvinylcaprolactam (LCST 35ºC).[43,44]

The possibility of applying TRLC in heart-cutting 2D-LC (LC-LC) has recently been illustrated by Kanazawa *et al.*, where a TR column was used as a pretreatment column.[45] In the present work, the potential of LC×LC is demonstrated, where TR columns are coupled to RPLC. The characteristics of TRLC permit the use of fully aqueous ^{1}D mobile phases, where solute retention is achieved by tuning the column temperature. The aqueous ^{1}D eluent has a very low eluotropic strength in the ^{2}D RP column of the second-dimension. This results in excellent on-column focusing at the head of the ^{2}D column, which allows for the use of broader ^{1}D columns and transfer of larger fraction volumes, thereby improving the sensitivity and peak capacity of the system.[46] To achieve this, poly(N-isopropylacrylamide) (PNIPAAm)-based columns were developed and implemented in a 2D-LC platform, after which the potential of the TRLC×RPLC combination was explored using complex mixtures of several standard compounds.

Experimental

Chemicals and reagents

Acetonitrile (ACN, HPLC grade) was obtained from Sigma–Aldrich (Steinheim, Germany). Milli-Q grade water (18.2 mΩ) was purified and deionized in-house by a Milli-Q plus instrument from Millipore (Bedford, USA). Formic acid (FA) was supplied by Acros (Geel, Belgium).

The standard test mixture for TRLC×RPLC consisted of compounds with varying functional groups. Methoxy-, ethoxy-, butoxybenzene as well as propyl and butyl benzoate, were from Acros; methyl-, ethyl-, propyl- and butylparaben, propriophenone, acetophenone and benzophenone were from Sigma-Aldrich, and n-hexanophenone and n-butyrophenone were from Janssen Chimica (Beerse, Belgium). Stock solutions of 1 or 2 mg/mL were prepared in ACN, according to the solubilities of the components. A mixture of all components was then prepared in acetonitrile/water (40:60) in concentrations ranging from 5 to 50 µg/mL, according to their relative absorbance at 254 nm.

The steroid mixture comprised methylprednisolone, cortexolone, hydrocortisone, hydrocortisone acetate, cortisone 21-acetate, testosterone and methyltestosterone, all supplied by Sigma–Aldrich as well as triamcinolone acetonide, supplied by Steraloids (Newport, USA). Stock solutions were prepared in ACN and the sample for analysis was prepared in acetonitrile/water (45:55) with the concentrations ranging from 45 to 90 µg/mL. A detailed overview of sample compositions is provided in the supporting information (Table S-3).

Chromatographic instrumentation and data analysis

The TRLC×RPLC instrument was assembled from two Agilent 1100 systems (Agilent Technologies, Waldbronn, Germany), interfaced via a two-position/ten-port switching valve with a micro-electric actuator (VICI, Houston, USA, model C2H-2000EH). The ^{1}D separation was performed using an 1100 quaternary pump equipped with a 1100 degasser coupled to an external six-port injection valve (Rheodyne, Alsbach, Germany). Isothermal temperature control of the column was provided by a temperature-controlled water/glycol bath (Julabo, Seelbach, Germany, model F10) and a 1100 variable wavelength detector (VWD) equipped with micro flow cell was used to monitor the ^{1}D separation. The ^{2}D instrument consisted of an 1100 binary pump, 1100 degasser and 1100 VWD equipped with a standard flow cell. All modules were controlled using two computers equipped with Chemstation software (Agilent). The first was used to control the ^{1}D pump, ^{1}D detector, ^{2}D detector and the second computer was used to operate the ^{2}D gradient on the ^{2}D pump. Raw data were exported as comma-separated values and converted to a data matrix in GC image R2.5 software (GCimage, Lincoln, U.S.A.). From these matrices

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contour plots were generated using OriginPro 8.5 (OriginLab Corporation, Northampton, U.S.A.).

<div align="center">Chromatographic conditions</div>

In the [1]D two 100 × 2.1 mm, 5 µm TR columns coupled in series were used, operating at a flow of 0.1 mL/min in isocratic mode. As mobile phase 0.1 vol% aqueous FA was used and the temperature of the column was controlled at either 25, 45 or 55ºC. Manual injection was performed with a sample loop of 20 µL and the separation was monitored by the [1]D VWD detector at 254 nm. For the [2]D separation, a 50 × 4.6mm, 3.5µm X-Bridge C18 column (Waters, Milford, U.S.A.) was used at room temperature. A flow of 5 mL/min was applied and the separation was registered by the [2]D VWD detector at 254 nm. As [2]D mobile phase, (A) 0.1 vol% aqueous FA and (B) ACN were used according to the following gradient program: 0 min: 0% B; 0.1 min: 30% B; 0.8 min: 35% B; 0.9 min: 50% B; 1.1 min: 60% B; 1.3-1.5 min: 100% B; 1.51 min: 0% B. The 10-port switching valve was equipped with two 200 µl loops, and the modulation period was 2 min.

Results and discussion

<div align="center">Development of [1]D TRLC separation</div>

The synthetic procedure adapted from reference [47] together with the characteristics of the TR polymer and of the obtained packing material is described in the supporting information (Section 1). In short, the polymer was obtained through free radical polymerization of NIPAAm, wherein 3-mercaptopropionic acid was used as a chain transfer reagent to introduce a carboxylic end group. This end group was then activated to form an active ester, allowing subsequent coupling to 5 µm aminopropyl silica particles.[47] 2.1 mm columns were packed to allow operation in the [1]D at 100 µL/min in the vicinity of the optimal flow rate.[48]

The temperature-responsive behavior of these columns is confirmed in Figure 1, where 4 parabens are analyzed at varying temperatures with water as mobile phase on two coupled TR columns. Significant increases in retention are observed upon increasing the temperature. Note that this effect in itself is remarkable, as Van't Hoff behaviour on conventional phases dictates a decrease in retention with temperature as a rule.[49]

<div align="center">Extrapolation TRLC×RPLC</div>

The proof of concept is illustrated in Figure 2, where the same TR separation as in Figure 1 is now used as first dimension in a TRLC×RPLC system. In this case, 200 µL fractions were alternatingly collected and injected on a [2]D RP column operated at a high flow rate. It is evident from this figure that, despite the large volumes injected onto the [2]D column, excellent peak shapes were obtained in the [2]D. The high permeability of the 50 × 4.6 mm, 3.5 µm [2]D column used here provide an average peak capacity of about ~21, a reasonable number when taking the relatively short gradient time (tg) of 1.5 min into consideration.[50] The peak capacities for the [1]D and TRLC×RPLC separations were ~12 and of ~226 respectively (calculations are included in the supplementary data). In the calculation of the [2]D peak capacity, a correction for the undersampling was taken into account according to[51], but no correction for orthogonality was made, as assessment of this aspect requires analysis of a broad range of solutes under different conditions in TRLC×RPLC, which is outside the scope of the current proof-of-principle contribution.[52] Although peak capacities of both [1]D and [2]D separations are relatively limited, the multiplicative effect in LC×LC is apparent, illustrating the potential of this particular column combination.
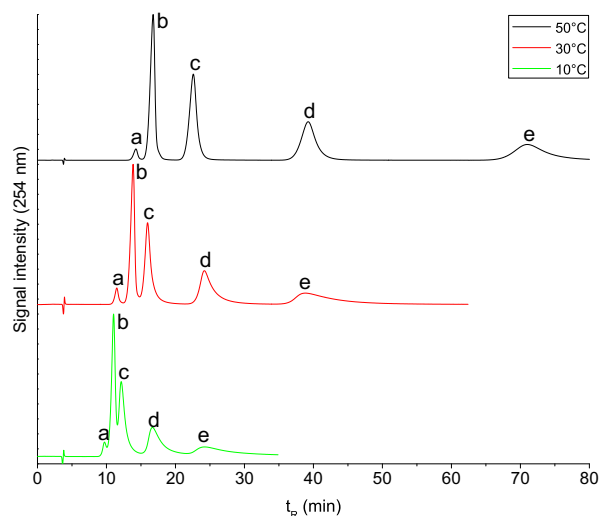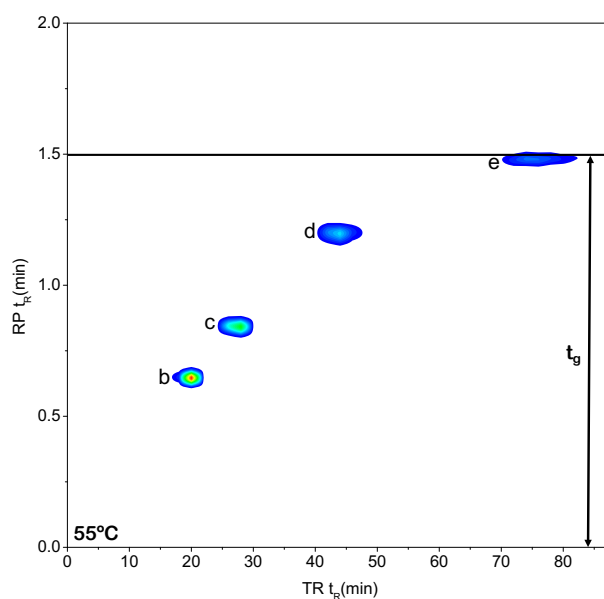

Figure 1. Example of a temperature controlled separation on coupled PNIPAAm-aminopropylsilica columns (200 × 2.1 mm, 5 µm), at 0.25 mL/min using 0.1 vol% aqueous FA as mobile phase. Compound labels: a) 4-hydroxybenzoic acid, b) methylparaben, c) ethylparaben, d) propylparaben, e) butylparaben (100 µg/mL each, injection volume 20µl).

Figure 2. Proof of principle TRLC×RPLC separation of parabens. [1]D TRLC separation operated at 55ºC. Other experimental conditions as specified in experimental section. Compound labels: b) methylparaben, c) ethylparaben, d) propylparaben, e) butylparaben (100 µg/mL each, injection volume 20µl).
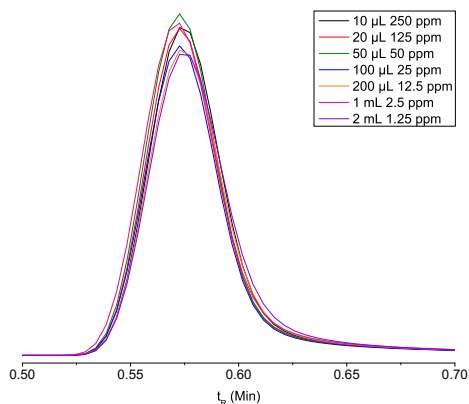
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Figure 3. Peak shape obtained for identical masses of aqueous methylparaben injected, in different volumes, directly on the ^{2}D column. Data are time corrected for the change in path length.

Evidence of on-column refocusing

The potential benefits of the proposed TRLC×RPLC methodology relies effective on-column refocusing when transferring the aqueous fractions from the TR column to the RP columns used in the ^{2}D. This principle was evaluated by injecting various concentrations of the least retained component (methylparaben) into the ^{2}D column operated under the same conditions, while keeping the injected mass constant by altering the injection volume. The results are represented in Figure 3, which shows that peak shapes are independent of the injected volume, confirming that on-column refocusing is extremely efficient under these conditions. Noteworthy is that this test reveals that virtually no loss in performance or sensitivity is observed for sample volumes up to 2 mL. This means that the fraction volume, and therefore the ^{1}D flow rate, can now be freely selected without the detrimental consequences of injection effects. As a consequence, the choice of ^{1}D column diameter is much less restrained in TRLC×RPLC method development, while sampling times also can be selected based on under-sampling criteria only, and not the effect of ^{1}D fraction volumes.

Assessment of the orthogonality of TRLC

As described in the introduction, the orthogonality is an important requirement of any LC×LC method to maximize utilization of the available two-dimensional separations spaces. As both the retentive mechanisms of conventional RPLC and of TRLC (when operated above the LCST) are largely based on hydrophobicity, a lack of orthogonality represents a concern for their combination, which is evident from Figure 2. In order to evaluate this aspect, the retentive behavior of both the temperature-responsive and reverse-phased columns were investigated and compared for selected analytes. This was done by comparing the retention factors of the selected compounds on both colums (represented in figure 4). This figure shows that both columns show a similar increase in retention with hydrophobicity within a linear series of the same analytes, confirming the previously made observations of figure 2. However, when looking at the retention behavior between the different analyte classes, a significant difference in retention is present based on the polar functional groups. This points to a significant polar component (reminiscent of polar embedded RP columns) effecting the retention in TRLC. From this it can be concluded that, although both separations are dependent on the hydrophobicity of the analytes, the additional retention of polar functionalities TRLC inherently introduces the requisite orthogonality.
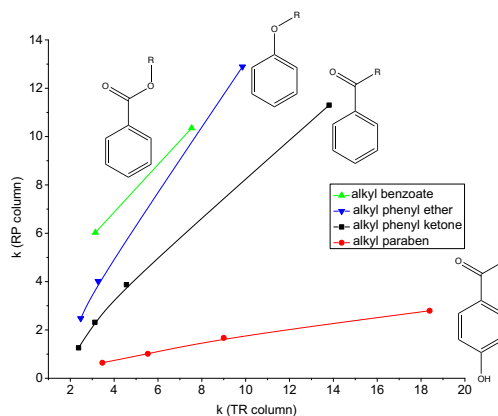

Figure 4. Representation of the correlation between the retention factors of the selected standard compounds on the TR and RP columns. The retention factors on the TR column were measured at 55ºC using 0.1 mL/min of 0.1 vol% aqueous FA as mobile phase, and the k values on the RP column were measured at 1 mL/min using 0.1 vol% aqueous FA and 0.1vol% FA in ACN at a 50/50 ratio. Both retention factors were measured on the respective columns used in the 2D setup. Alkylparaben series: methylparaben, ethylparaben, propylparaben, butylparaben; alkylphenylketone series: acetophenone, propriophenone, butyrophenone, hexanophenone; alkylphenylether series: methoxybenzene, ethoxybenzene, butoxybenzene; alkylbenzoates: propylbenzoate, butylbenzoate

TRLC×RPLC applications

The performance of this new LC×LC approach is demonstrated through the analysis of a test mixture containing analytes spanning a range of polarities (Table S-1). The separation of the mixture of parabens, alkoxybenzenes and phenones is represented in Figure 5, where the TRLC column was operated at both 25°C and 55°C. Significant improvements are obtained for the separation at 55ºC, mainly due to the increased retention and faster mass transfer in TRLC at higher temperatures. The slight waviness of the compound peaks is caused by the small difference in path lengths caused by the jumperloop on the 10-port valve. Several solutes pairs which might be challenging to separate in the individual dimensions are separated in TRLC×RPLC without requiring any method development. The complementary retention information obtained by TRLC and RPLC described above is visually confirmed: the hydrophobic solutes containing more polar functionalities, such as the parabens, now occupy a different zone in the lower section of the contour plots compared to the purely hydrophobic analytes visible at the top. As no solutes can elute before the void time (0.4) min in ^{2}D, a fair coverage of the available separation space is achieved. In contrast, when TRLC is performed below the LCST, a large drop in retention and in separation quality is observed. Although the phase now displays polar functionalities, this does not seem to lead to useful retention of the more polar solutes in this sample. This is most likely due to the high eluotropic strength of the aqueous mobile phase in what is essentially a HILIC or normal-phase type separation under these conditions. Therefore, the main benefits of using of lower temperatures, also in TRLC×RPLC, seems to be the possibility of peak focusing it offers in ^{1}D together with the potential for tuning of the retention, when using reverse temperature gradients in TRLC. For comparison, TRLC×RPLC was also applied to the analysis of a mixture of steroids (Figure 6). Although peak broadening is severe for such solutes in TRLC, the combined TRLC×RPLC setup allows for baseline resolution of most solutes through the combination of both separations.
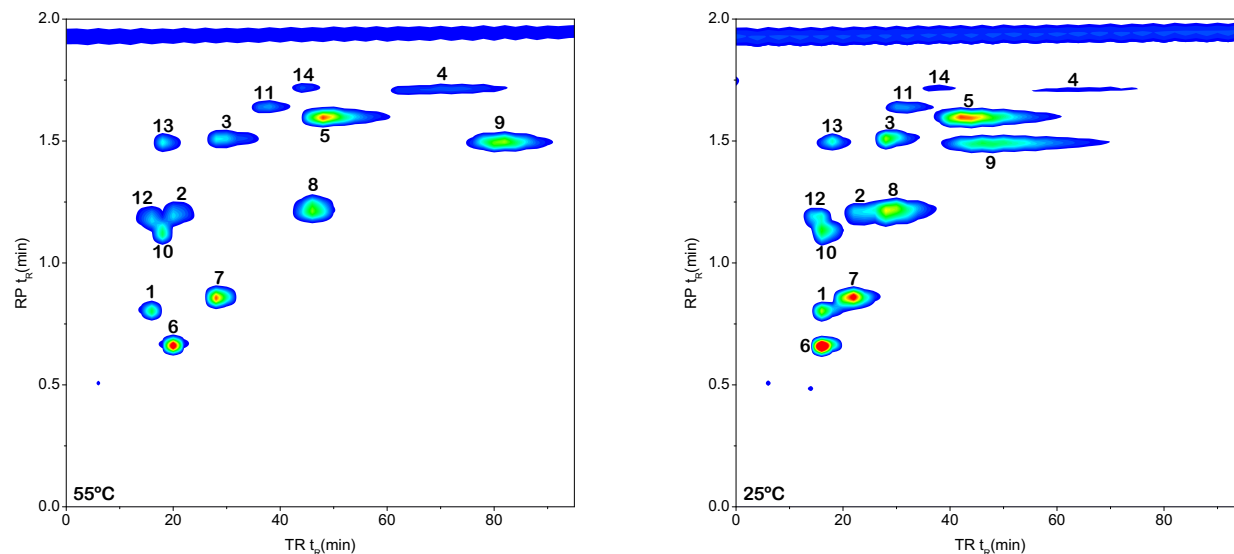
Figure 5. Contour plots at 254 nm obtained for the TRLC×RPLC separation of a test mixture, with the ¹D TRLC separation operated at 55ºC (left) and 25ºC (right). Other experimental conditions as specified in experimental section. Compound labels: 1) acetophenone, 2) propriophenone, 3) butyrophenone, 4) hexanophenone, 5) benzophenone, 6) methylparaben, 7) ethylparaben, 8) propylparaben, 9) butylparaben, 10) propylbenzoate, 11) butylbenzoate, 12) methoxybenzene, 13) ethoxybenzene, 14) butoxybenzene

Conclusions

In this work, the combination of TRLC and RPLC in the first and second dimensions of an LC×LC platform was demonstrated, offering a novel approach to address the problems associated with modulation.

The key features of the approach are:

- Problem-free modulation is achieved as a consequence of complete focusing of aqueous fractions of the ^{2}D, resulting in no loss of resolution when the sample volume is altered and thereby circumventing the need for more complex interfacing techniques.
- Sufficient orthogonality for the system was hypothesized by direct comparison of the retention behavior of the TRLC to the polarity of the analytes and experimentally demonstrated though the analyses of text mixtures.

Currently, the realized peak capacities are still limited by the low efficiencies in both dimensions, although this can be remedied by using UHPLC instrumentation in the ^{2}D and further optimization of the TR column manufacturing strategies to improve the efficiencies of these columns. Next to these improvements in peak capacity, optimization of the separation space can achieved through the introduction of shifted gradients in the 2D.[53]

This new LC×LC approach offers novel possibilities for the separation of complex mixtures of organic molecules. The efficient on-column refocusing inherent to the combination of TRLC and RPLC alleviates restrictions placed on the ¹D column diameter and simplifies modulation. Further exploitation of these benefits would then allow for the development of techniques such as 2D-prep-LC, improved LC×LC-MS methods and LC×LC methods with a higher detection sensitivity.

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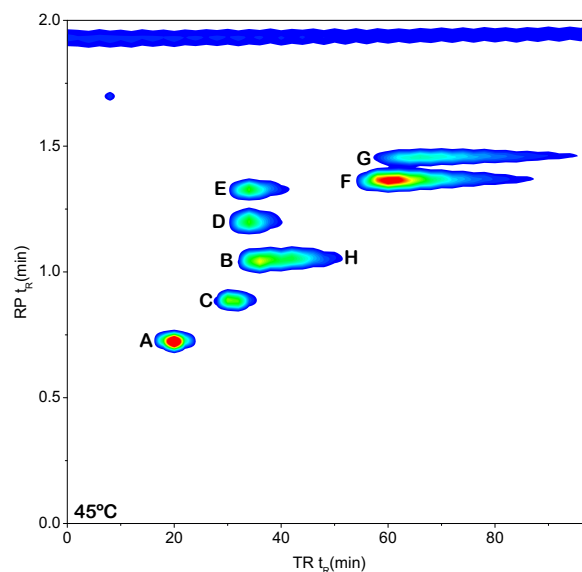E-mail: frederic.lynen@ugent.be

Figure 6. Contour plot obtained at 254 nm for the TRLC×RPLC separation of a steroid mixture. ¹D TRLC separation operated at 45ºC. Other experimental conditions as specified in experimental section. Compound labels: A) methyl-prednisolone, B) cortexolone, C) hydrocortisone, D) hydrocortisone acetate, E) cortisone 21 acetate, F) testosterone, G) methyl-testosterone, H) triamcinolone acetonide

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