Perspectives in hydrophobic interaction temperature-responsive liquid chromatography (TRLC)

Frédéric Lynen[1], Adriaan Ampe[1], Elena Bandini[1], Mathijs Baert[1], Kristina Wicht[1], Ardiana Kajtazi[1], Turaj Rahmani[1], Jonas Veenhoven[1], Gaëlle Spilleers[1].

[1] Separation Science Group, Ghent University, Krijgslaan 281 S4-bis, 9000 Ghent, Belgium

* Corresponding Author:
e-mail address: Frederic.Lynen@UGent.be
tel. : +32 09 264 96 06
Fax. : +32 09 264 49 98

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Abstract

Temperature responsive liquid chromatography (TRLC) is an emerging green HPLC mode allowing reversed phase type separations while necessitating only water as mobile phase. The columns therein are typically packed with silica particles to which stimuli-responsive polymers are anchored. In hydrophobic interaction TRLC, such polymers depict a loss of water solubility when increasing the temperature above a characteristic conversion temperature, causing large changes in retention over quite narrow and mild temperature ranges (~ 5-55°C). TRLC circumvents the concerns about analyte or column degradation which can occur when implementing high temperatures (> 80°C) on conventional reversed phase columns. It allows for HPLC using only water often spiked with the additives typically used in reversed phase LC. Therefore, this separation mode allows for greener, cheaper and isocratic analyses under non-denaturating conditions. The absence of compositional solvent gradients also allows for the exploitation of temperature gradients in combination with refractive index detection. Purely aqueous hydrophobic interactions TRLC is mostly applicable for solutes depicting a $1 < LogP < 5$, yet these ranges can be expanded through implementation of combined aqueous/organic mobiles phases, while preserving the temperature-responsive effects. In this first TRLC instalment our recent developments, new possibilities and current limitations of the use of 1-D TRLC are discussed, whilst the column performance is described with respect to the fundamentals of HPLC.

Introduction

Temperature-responsive liquid chromatography (TRLC) is an emerging separation mode in liquid chromatography allowing obtaining reversed phase type of separations under purely aqueous conditions. In this mode, retention is controlled through the column temperature while the mobile phase is invariable and composed of only water. This stands to contrast with conventional reversed phase liquid chromatography (RPLC) performed on e.g. octadecylsilica (C18) whereby compositional solvent gradients are usually necessary to allow timely elution of disparate molecules in terms of polarity and retention. Hence TRLC avoids the need for organic co-solvents in the aqueous mobile phase.

The approach has mainly been based on the exploitation of a particular class of stimuli-responsive (often dubbed "smart") polymers which lose their water solubility once a certain temperature is exceeded. The temperature at which this transition occurs depends mainly on a lower critical solution temperature (LCST) which is characteristic for each polymer. However, the molecular weight, concentration and (poly)dispersity also influence the experimental polymer precipitation temperature. In TRLC, the polymer chains are typically anchored to silica particles and packed into stainless steel HPLC columns. The concept was introduced by Kanazawa et al. in the early nineties followed by a variety of demonstrations and enhancements, including by our group, in the last two decades (1-8).

Temperature-responsive polymers depict a drastic change in their physical properties with temperature. In general, such behavior in polymers is not uncommon, providing one can consider any solvent composition. As only a limited number of polymers are responsive in water and around physiological temperatures, these have attracted most attention for applications such as cell culture release, drug delivery and for their potential in liquid chromatography (9, 10). This phenomenon has mostly been studied in substituted polyacrylamides with particular emphasis on poly(N-isopropylacrylamide) (PNIPAAm). The utilization of the ensuing stationary phases in LC allows obtaining increased or reduced retention at higher or lower temperatures, respectively as illustrated in Figure 1. This is caused by the formation of an apolar layer on the surface of the stationary phase owing to the precipitation (demixing) of the polymer from the aqueous mobile phase at higher temperatures. Correspondingly, cooling down the column resolubilizes the polymer chains which is concomitantly eliminating their retentive ability. The retention mechanism is based on hydrophobicity and is therefore similar to RPLC. However, it allows operation with only water as mobile phase whereby the retention increases drastically with mild increases in temperatures. In this instalment the possibilities and limitations of TRLC are discussed with respect to the chromatographic fundamentals.

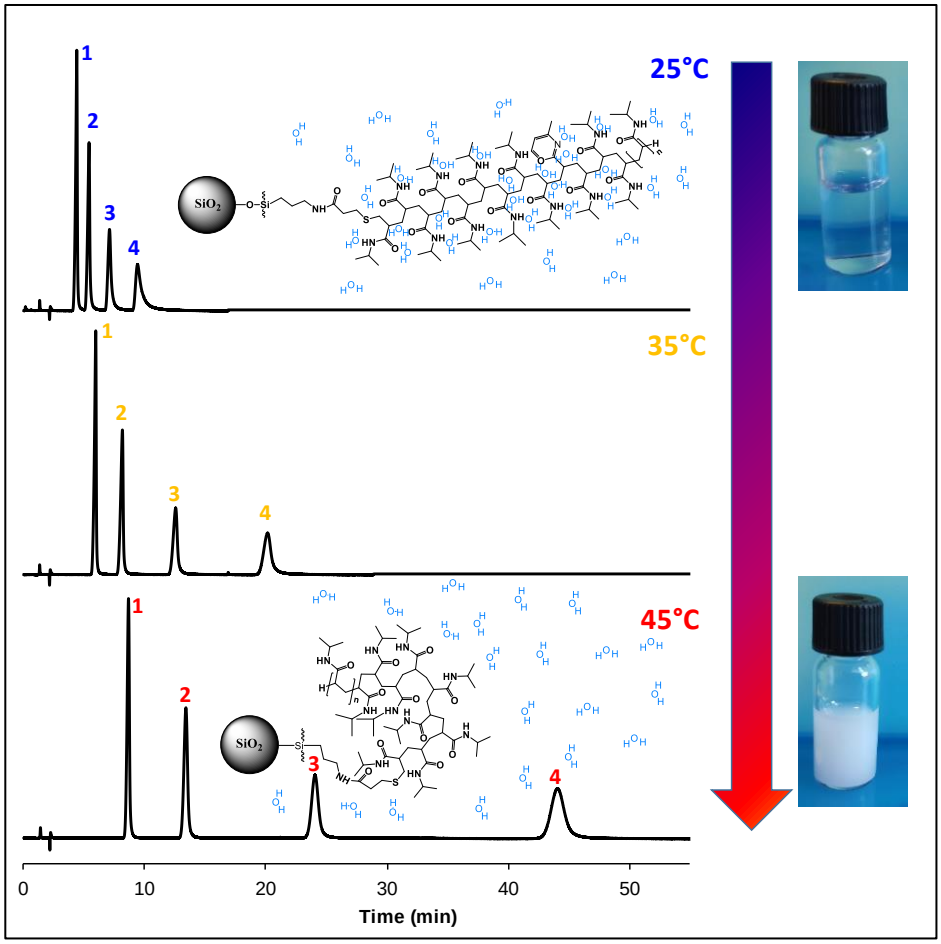

Figure 1: TRLC separation of methyl- (1), ethyl- (2), propyl- (3) and butylparaben (4) using water as mobile phase. flow rate 0.25 mL/min; column: poly(N-isopropylacrylamide) based, 4.6 mm x 150 mm (5 µm d_p). Conditions as in (8).

Retention

Thermodynamics of retention

In RPLC, one typically observes a decrease in retention when increasing the temperature. Thermodynamically this means that retention is enthalpically ($\Delta H_{retention}$) favorable and entropically ($\Delta S_{retention}$) unfavorable (11). As chromatographic retention is usually exothermic, the Gibbs free energy equation ($\Delta G_{retention} = \Delta H_{retention} - T\Delta S_{retention}$) shows that when increasing the temperature, the retention phenomenon becomes less favorable (as $\Delta G_{retention}$ is becoming less negative at elevated temperatures). This can be represented via a Van 't Hoff plot (ln k vs 1/T) as shown e.g. for Linuron in Figure 2, whereby it can be seen that indeed the retention is slowly decreasing with increasing temperature on a C8 column.

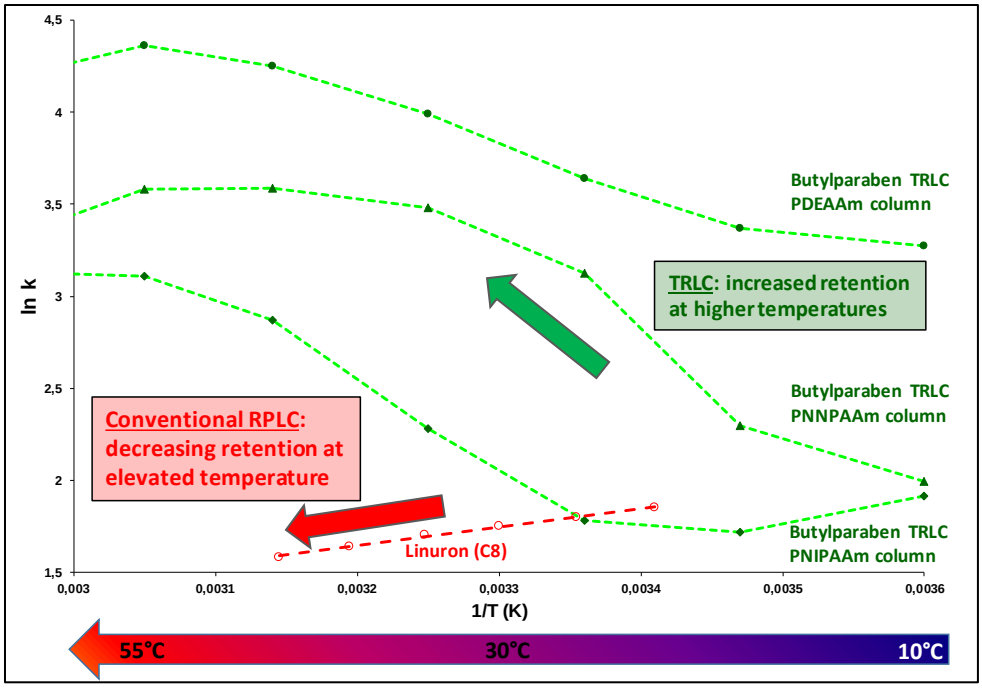

Figure 2: Van 't Hoff plots (ln k vs 1/T) obtained for butyl paraben data obtained on various TRLC columns (4) with water as mobile phase. Comparison with representative RPLC retention data observed on a C8 column (12).

In TRLC, this retention behavior is drastically altered because the stationary phase itself (and hence mainly $\Delta H_{retention}$) undergoes a radical, reversible change from a dissolved, highly hydrated random coil to a more condensed globular shape. Thermodynamically, this conversion is entropically driven. The enthalpy contribution of dissolving the polymer in water is exothermic ($\Delta H_{polymer\ dissolution} < 0$) due to the formation of favorable hydrogen bonds between the water molecules and the polar groups of the polymer leading to hydration of the polymer. However, dissolving macromolecules with apolar groups on the backbone and side-chain causes a significant loss in entropy ($\Delta S_{polymer\ dissolution} < 0$), as the water molecules are forced into a cage-like orientation around these hydrophobic parts of the polymer (see Figure 1). As the temperature increases, the enthalpy contribution becomes weaker and the entropic loss becomes more pronounced leading to a positive Gibbs free energy ($\Delta G_{polymer\ dissolution} = \Delta H_{polymer\ dissolution} - T\Delta S_{polymer\ dissolution}$), which causes the dehydration of the polymer chains. Before the dehydration, the hydrophobic side chains on the polymer are diffuse and spread over a larger volume providing little retention. After the dehydration the polymer exposes dense hydrophobic zones allowing for enlarged retention based on hydrophobicity.

It is interesting to observe that when the polymer is fully hydrated under cold conditions or fully precipitated in a warm environment, that further temperature change beyond these points causes the normal thermodynamic behavior to take (back) the upper hand. This is e.g. also visible through the chromatograms represented in Figure 3 obtained for a number of TRLC columns based on various polymers.

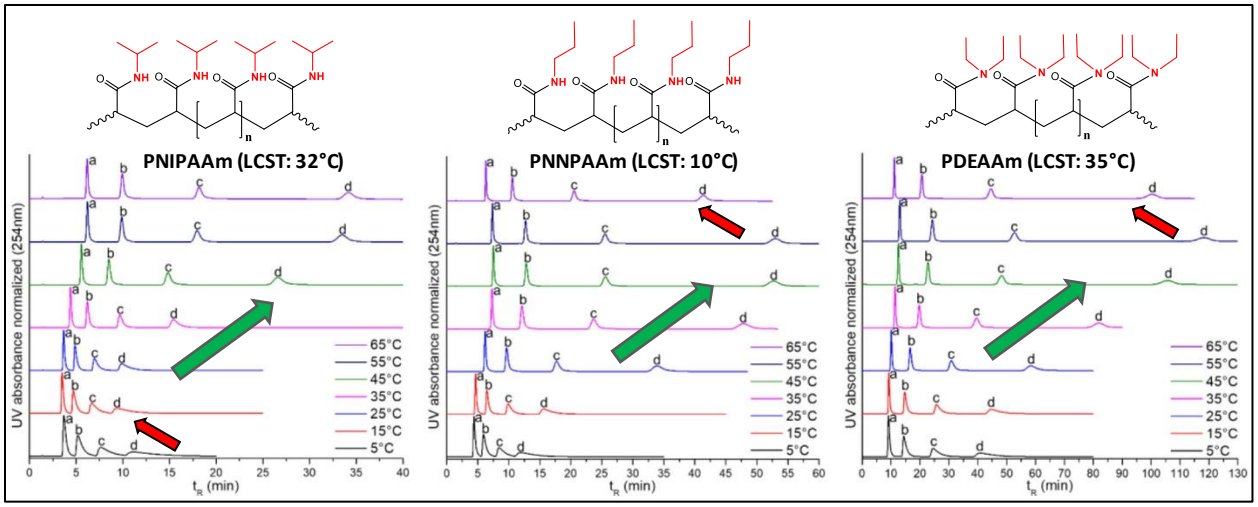

Figure 3: Temperature-responsive effect observed on the TRLC columns (4.6 mm x 100 mm x 5 µm d_p) obtained on three TRLC column types for the separation of parabens. Water was used (+0.1% formic acid) as mobile phase at a flow rate of 1.0 mL/min. Peak identification: (a) methylparaben, (b) ethylparaben, (c) propylparaben, (d) butylparaben. The N-substituted polyacrylamides types were poly[N-isopropylacrylamide] (PNIPAAm), (poly[N-n-propylacrylamide] (PNNPAAm) and poly[N,N-diethylacrylamide] (PDEAAm). Red arrow: conventional thermodynamic behavior causing a decrease in retention with temperature. Green arrow: TRLC based increase in retention with increasing temperatures Figures modified with permission from (4).

Other aspects controlling retention in TRLC

Chromatographically one aims for retention factors (k) >3 and <10 as this maximizes resolution while avoiding excessive analysis times. As illustrated in Figure 2 and 3, the use of different polymers (on otherwise identical columns) leads to different retention time changes as a function of temperature. When the isopropyl group in the most used PNIPAAm columns is altered for a linear n-propyl chain, the conversion temperature in the resulting PNNPAAm column drops to lower temperatures while larger retention is obtained for the parabens compared to the first column type. From an HPLC point of view, the ability to perform TRLC at even lower temperatures could become useful because of the related diminished potential issues in terms of analyte stability and silica hydrolysis. When the single propyl groups are replaced by two ethyl groups per monomer, a more strongly retentive column is obtained, which might offer promise for applications where the PNIPAAm columns offer too little retention (e.g. for biomolecule analyses). A current limitation of TRLC is that even at the lowest retentive conditions on all TRLC column a residual retention is obtained. Research has also been performed based on the implementation of co-polymers, whereby in most cases one of the monomers depicted TRLC behavior and the other allowed for enhanced hydrophobic or Coulombic retention. While copolymers can allow for e.g. better biomolecule retention, it also often further increases the residual column retention while reducing the range of retention factors which can be effectively covered through TRLC (1). As the latter is already currently not large in TRLC (see e.g. figure 3A where k varies from 7 to 23 on a PNIPAAm column for butylparaben) the incorporation of sections in the polymer which do not depict temperature responsive behavior diminishes the range of retention which can be covered with a column. Conversely the combination of two or more temperature-responsive polymers in a column might be promising for being able to exploit a broader reachable retention time window. Various studies have also reported the use of cross-linked networks as compared to linear polymer strands. While again promising applications have been shown this doesn't' seem to offer the prospect of generically applicable columns as they offer a more limited span of reachable retention factors (9).

Next to temperature and polymer composition, the retention also proves dependent on the grafting density of the polymer brushes immobilized on the silica particles, the molecular weight, the polydispersity, the coupling chemistry and on surface characteristics of the supporting material. While these aspects can be controlled, an evolution towards narrower polymer dispersity immobilized on a highly controlled surface topology will allow further incremental improvement of TRLC. Most examples of successful TRLC indicate a carbon load on the silica > 15% whereby polymers depict between 40 and 50 repeating units with as narrow a distribution as possible. While obtaining a shift in retention with temperature is essential in TRLC, the magnitude of this shift can vary from a modest doubling of retention to a sometimes about tenfold increase. Ideally one would strive in TRLC to obtain a similar (extremely broad) ability to tune k from 0 to about infinite as is the case when using different solvent compositions in RPLC. While this is currently unachievable, the ability to control k even over a comparatively narrow order of magnitude already offers significant benefits in terms of gradient peak focusing and analysis time reductions (13, 14).

Unsurprising, the composition of the mobile phase also plays a strong role in the conversion of the polymer. The addition of salts favors the dehydration of polymers which lowers the dehydration temperatures and consequently enhances the column retention at a given temperature (8). A drawback thereof is that in order to obtain a useful influence, high (0.2-2 M) concentrations are required. While volatile salts can also be used, this is approach is nevertheless demanding for the instrumentation and columns. Alternatively, organic modifiers also strongly influence the polymer conversion temperature and the overall retention (3, 7). While they strongly decrease the retention, the addition of relatively small fractions (5-15 %) can be beneficial as it can remove (or reduce) the residual polymer retention at lower temperatures, offer enhanced efficiencies and elution of highly hydrophobic solutes which cannot elute with only water as mobile phase. Although this approach is contrary to the green incentive to develop TRLC, it might offer promise e.g. when combined with bio-sourced or biodegradable solvents.

Efficiency

In general polymer based columns are often perceived to offer only a limited column efficiency as compared to silica based columns (15). Because in TRLC rather short polymer strands are immobilized on silica, similar column efficiencies and plate heights can be expected to be reachable as obtained with RPLC columns. The best reported performances (for well retained solutes) in TRLC offer column efficiencies which are only 33% below the optimal performance reachable on C18 columns. Correspondingly the minimal reduced plate heights ($h = H/d_p$) are close to 3 (instead of 2 as obtained on a column packed with fully porous C18 particles). In Figure 4, the van Deemter plots obtained for the 3 TRLC columns described above (A) are represented as collected under the most retentive conditions (55°C) and compared with an earlier TRLC study (B) whereby a different (free instead of a controlled radical polymerization) synthetic route was followed.

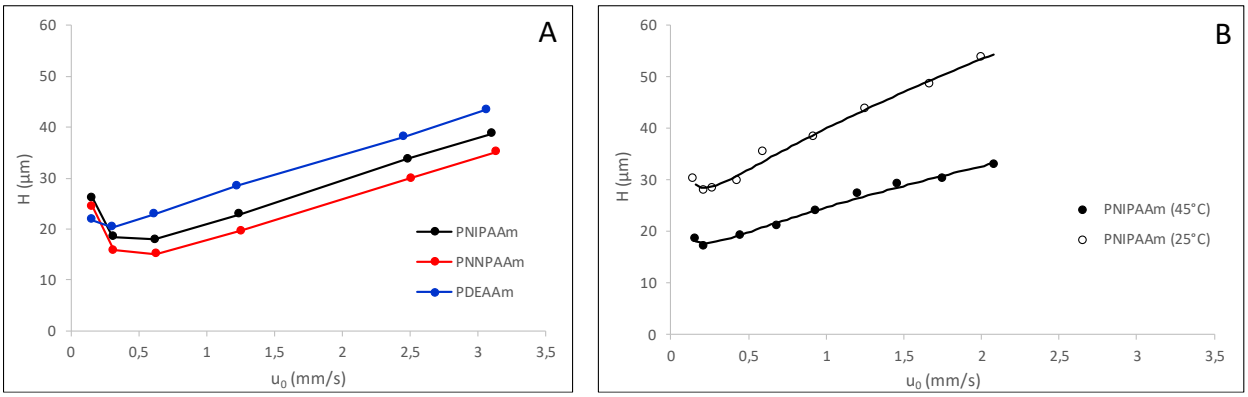

Figure 4: A: averaged plate heights obtained (in triplicate) for methyl-and ethylparaben on the PNIPAAm, PNNPAAm and the PDEAAm columns vs the linear velocity at 55 °C. Column dimensions: 10 cm x 4.6 mm, 5 µm d_p. Data adapted from (4). B: Comparative Van Deemter plot data obtained for triamcinolone acetonide on an PNIPAAm based column (15 cm x 4.6 mm, 5 µm d_p) (8).

While this illustrates that indeed reasonable and improvable efficiencies can be obtained in TRLC, it can also be seen that H/u plots are characterized by a rather steep C-term regime. This is attributed to the resistance to mass transfer in the stationary phase (C_s), which is inevitably larger in a bulky polymer layer as compared to a thin layer of C18 groups. As a consequence, the optimal linear velocity is found at about 3 to 4 times slower flow rates compared to conventional RPLC. Hence, in order to reach optimal chromatography in TRLC currently longer analysis times are still a necessity. Without doubt TRLC can become more efficient through the use of smaller fully or partially porous particles. The main hurdle therein has been the current practical unviability of sub-2 micron silica based supporting materials which offer a suitable target functional group to which the polymers can be covalently anchored. In most TRLC work the polymers are synthesized such as to contain a carboxylic end group, which can then be attached to aminopropylsilica via various amide bond forming chemistries (2, 4, 6, 8).
On the other hand, when TRLC is performed under the poorly retentive low temperature conditions, the polymer layer is extended and therefore more voluminous. This detrimentally affects the mass transfer (as shown in Figure 4B at 25°C for PNIPAAm) and the corresponding efficiencies obtained under those conditions. This is, however, unproblematic in terms of the applications as in most cases a downwards temperature gradient is used, causing the peaks to refocus (see section: gradient analyses).

Selectivity

The selectivity in TRLC is altered through temperature, polymer composition and through additives. While at lower temperatures some enhanced retention of polar solutes can be observed, this has not led to promising applications. This is in part related to the above mentioned poor efficiencies obtained at low temperatures. At temperatures above the LCST the retention is mainly based on hydrophobicity. When altering the polymer composition, different selectivities are observed, even for the 3 conceptually similar polyacrylamide. This is illustrated in Figure 5 whereby changes in elution order are apparent for the analysis of steroids and barbiturates. The different selectivities can also be combined via a Stationary-Phase Optimized Selectivity LC type of approach to deliver an optimized separation (16, 17). TRLC also proves able to separate closely related diastereoisomers as illustrated for a number of peptides in Figure 5C (6).

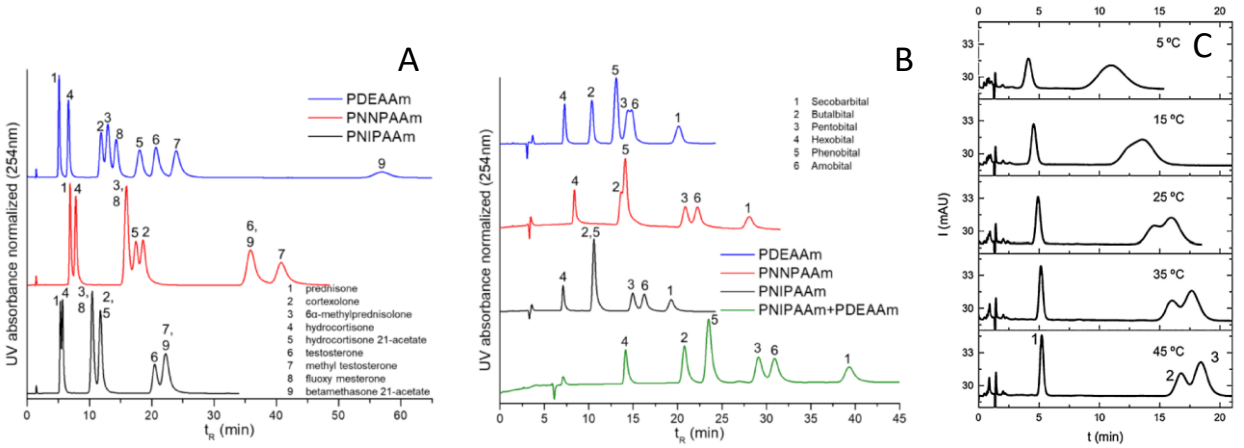

Figure 5: Analyses of a sample comprising 9 steroids (A) or 6 barbiturates (B) on three TRLC column types at 55°C in water (+0.1 % FA) at 1 mL/min(4). C: Analysis of a dipeptide and of two diastereoisomeric tripeptides on a PNIPAAm column at various temperatures (6). Peak identification: (1) AcNH-l-Tyr-d-Phe-CONH₂, (2) AcNH-l-Tyr-d-Phe-d-Phe-CONH₂ and (3) AcNH-d-Tyr-l-Phe-d-Phe-CONH₂.

Gradient analyses

Hydrophobic interaction TRLC is mainly operated at temperatures above the LCST, allowing exploitation of the then obtained higher retention, while benefiting of reasonable plate numbers and selectivity reachable under those conditions. The main benefits of TRLC can be reaped when this is combined with downwards temperature gradients imposed on the columns. Although the column temperature can also be programmed in conventional static air ovens, their thermal mass hinders imposing rapid cooling gradients. Programmable water baths are mostly used to control the column temperature in TRLC as they allow accurate temperature control. Recently, we introduced a column cooling approach based on the controlled mixing of a hot and cold mobile phase as reachable through any binary HPLC pump, allowing for faster gradients as compared to the use of programmable water baths (13). The ensuing drastic reduction in retention time and obtained gradient peak focusing are illustrated in Figure 6.

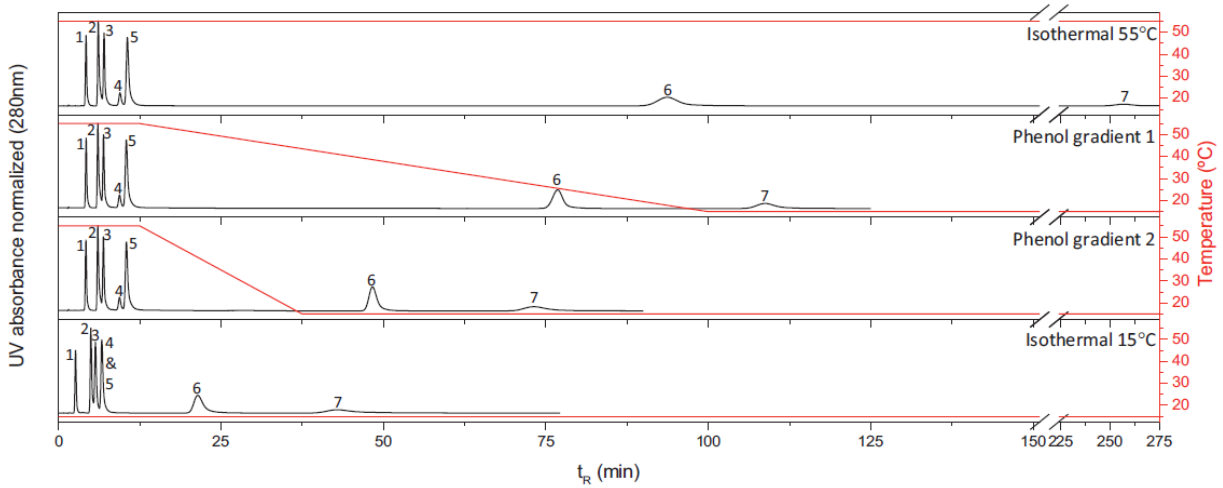

Figure 6: Gradient separations of a natural phenol mixture at a flow rate of 1 mL/min via an axial gradient HPLC system. The applied temperature gradients are represented in the graph (red overlay). Compound labels: (1) naringin, (2) gallic acid, (3) vanillic acid, (4) caffeic acid, (5) catechin, (6) resveratrol, (7) kaempferol. Reproduced with permission from (13).

The use of gradients also allows for novel possibilities in the field of quantitative analysis. While the combination of compositional solvent gradients with refractive index detection is prohibitive, the use of the thermal gradients required in TRLC proves not to influence the noise, drift or response of this detector. A quantitative application is shown for a series of short chain fatty acids determined under the steepest gradient conditions (45 to 5°C at -1°/min). The overlapping calibration lines illustrate that standard independent quantitation now become possible, while the applicability range of the RI detector is extended.

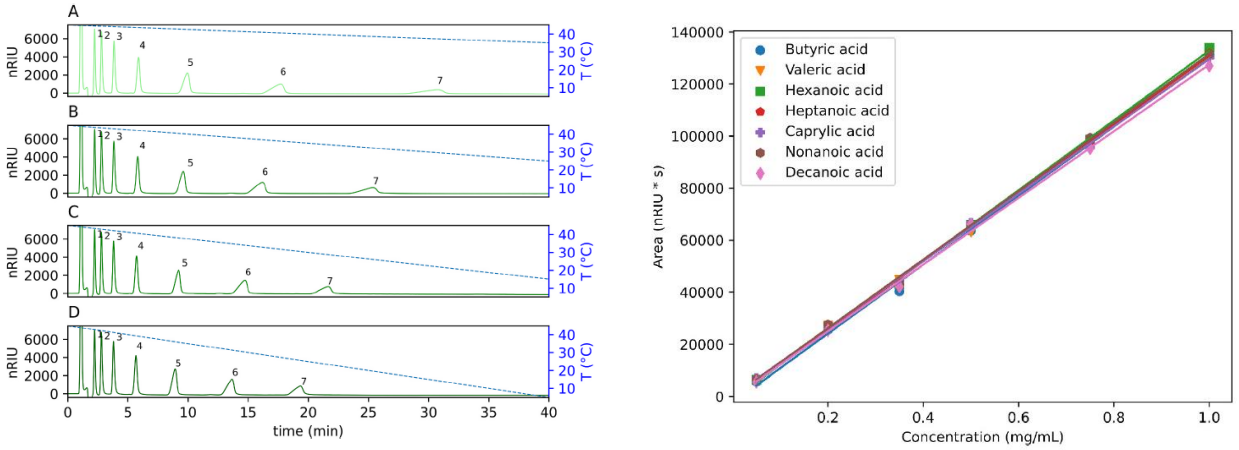

Figure 7: (left) Chromatograms obtained for the separation of fatty acids by TRLC-RID with varying temperature gradient profiles, -0.25 °C/min (A),-0.5 °C/min (B), - 0.75 °C/min (C), -1 °C/min (D). Mobile phase H2O + 0.1% Formic Acid, PNIPAAm column (5 cm × 4.6 mm, 5 µm dp). Peak identification: 1. butyric acid, 2. valeric acid, 3. hexanoic acid, 4. heptanoic acid, 5. caprylic acid, 6. nonanoic acid, 7. decanoic acid (0.5 mg/mL 1 each). (right) Calibration lines obtained at -1 °C/min for the 7 short chain fatty acids.

Summary

TRLC is an alternative separation mode allowing obtaining similar separation as in reversed phase LC while using only water as a mobile phase. Although poly(N-isopropylacrylamide) (PNIPAAm) is still the most used polymer in TRLC, the possibilities offered by other temperature-responsive acrylamides is appealing and potentially more interesting as they offer more retention or dehydrate at lower temperatures. The efficiency of the columns obtained via such polymers immobilized on silica particles appears not fundamentally hindered and an evolution towards more efficient columns appears realistic. Nevertheless, the bulky nature of a polymer layer on silica slows down the mass transfer and the resulting optimal velocities. The selectivity of TRLC columns differs between the polymers and at different temperatures. TRLC is most useful when it is used under thermal gradient conditions, which allow for peak focusing, and reduced analysis times. It could also be shown that the use of temperature gradients in TRLC is compatible with refractive index detection, allowing for novel applications via this fairly universal detector.

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