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Authors: **Bram Miserez, Frédéric Lynen, Adrian Wright, Melvin Euerby, Pat Sandra**

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Thermoresponsive poly(N-vinylcaprolactam) as stationary phase for aqueous and green liquid chromatography

[1]Bram Miserez, [1]Frédéric Lynen, [2]Adrian Wright, [3]Melvin Euerby, [1]Pat Sandra*

[1]Pfizer Analytical Research Centre, Ghent University, Krijgslaan 281 S4-bis, 9000 Ghent, Belgium

[2]Structural and Separation Sciences, Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent, CT13 9NJ, United Kingdom

[3]Hichrom Ltd., 1 The Markham Centre, Station Road, Theale, Reading, Berkshire, RG7 4PE, United Kingdom

Abstract

Poly(N-vinylcaprolactam) (PVCL) connected to aminopropyl silica is a new stationary phase for temperature responsive liquid chromatography (TR-LC). PVCL shows a transition from hydrophilic to hydrophobic interaction between 30 and 40°C. The synthesis is described in detail. The temperature responsive characteristic of the phase is illustrated with a mixture of steroids using pure water as mobile phase. An increase in retention is observed when raising the temperature. Hu-plots at different temperatures were constructed. Below the lower critical solution temperature (LCST), no optimal velocity could be measured because of substantial resistance to mass transfer indicating an adsorption mechanism. Above the LCST, partitioning controls the separation resulting in higher efficiencies and an u_{opt} of ca. 0.3 mm s^{-1}. Reduced plate heights decreased from 4 at 45°C to 3 at 65°C. The temperature responsive nature of the polymer is lost in green chromatography with ethanol as modifier in concentrations above 5%.

Key words

Thermoresponsive LC, poly(N-vinylcaprolactam), aqueous and green mobile phases.

Correspondence

Prof. Dr. P. Sandra

PARC-UGhent

Krijgslaan 281-S4bis

B-9000 Gent

Belgium

Tel: +32 56 204031

Fax: + 32 56 204859

e-mail:pat.sandra@richrom.com

Introduction

In a previous paper, we described the synthesis and chromatographic characterization of the temperature responsive stationary phase poly(N-isopropylacrylamide) (PNIPAAm) [1]. The rationale behind this project was linked to the development of an on-line high-performance LC/continuous flow biochemical screening assay system. The basic principle of such a system was described by de Boer et al. [2]. One of the problems in the construction of generic on-line LC/bioassay methods is the application of reversed-phase LC in which relatively high concentrations of organic modifiers are applied decreasing or destroying the activity of enzymes. A way to circumvent partly the problem was the application of elevated temperature LC through which the modifier concentration could be drastically reduced [3]. However, concerns related to compound and stationary phase stability at elevated temperatures could limit the applicability of this approach. One of the advantages of temperature responsive LC (TR-LC) on PNIPAAm is that pure water can be applied as mobile phase. This has already been illustrated in the pioneering work on TR-LC by the group of Okana et al. [4-6] and in many applications including the analysis of small molecules, pharmaceuticals and biomolecules [7,8]. Columns containing copolymers of NIPAAm with hydrophobic [5-7, 9], ionic [8,10], and affinity ligands [11] have extended the applicability range of TR-LC.

Polyacrylamides have been favoured so far in temperature responsive applications because of their rapid change in hydrophobicity over a very narrow temperature range. The chromatographic application of thermoresponsive polymers, however, does not necessarily require a fast transition between the hydrophilic and hydrophobic state as a function of temperature. Poly(N-vinylcaprolactam) (PVCL) is such a polymer showing a more gradual change from hydrophilic to hydrophobic upon raising the temperature. The polymer has an LCST between 32 and 37 °C and exhibits a different dependency of the demixing temperature on the corresponding concentration and chain length when compared to PNIPAA [12,13]. This is, however, not expected to influence the chromatography much as, just for the latter, an increase in temperature will still lead to an increase

in retention. Notice that PVCL has a syndiotactic structure, in this way also differing from PNIPAAm which has an atactic structure. PVCL has been studied in solution [13], as a copolymer [14], in hydrogels (hydrophilic network) [15,16] and bound to carriers [17]. It has been applied for cell and enzyme entrapment [15], as a catalyst support [18] and, combined with peptides, for wound healing [19]. It has also been used to control pore openings in track etched membranes [20]. PVCL has been coupled to silica and the use of this material for chromatography has been hinted [21], but no reports have been published. In this contribution, the preparation of a silica stationary phase containing poly(N-vinylcaprolactam) is described. Columns packed with PVCL were evaluated in the temperature range 15 to 65°C with water and with water/ethanol as mobile phase.

Experimental

Chemicals

Aminopropyl silica (Nucleosil 100-5 NH_2) was purchased from Macherey Nagel (Düren, Germany). The following chemicals were used for the synthesis and obtained from Sigma-Aldrich (Bornem, Belgium): 4,4'-azobis(4-cyanovaleric acid), mercaptopropionic acid, N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), and N-vinylcaprolactam (VCL). HPLC grade water, methanol (MeOH), acetonitrile (ACN), isopropanol and dimethylformamide (DMF) were obtained from Sigma-Aldrich. DMF and mercaptopropionic acid were distilled under reduced pressure before use. VCL was re-crystallized from benzene and dried under vacuum. The steroid mixture was composed of hydrocortisone, prednisolone, cortisone, cortexolone, hydrocortisone acetate and methylprednisolone; the paraben mixture included methylparaben, ethylparaben, n-propylparaben and n-butylparaben; and the phenone mixture acetophenone, propiophenone, valerophenone, benzophenone, hexanophenone, heptanophenone and octanophenone. All standards were from Sigma-Aldrich.

Final synthesis

10 g aminopropyl silica, 15.5 g EEDQ (62.5 mmol) and 8.7 g 4,4'-azobis(4-cyanovaleric acid) (31.0 mmol) were reacted in 125 mL DMF. After 16 h gentle swirling under nitrogen, the silica was filtered and washed with 250 mL water and 250 mL MeOH and dried under vacuum. 10 g of this silica and 15 g of VCL (107.8 mmol) were added to 125 mL of DMF and heated to 90°C for 16 h under nitrogen and gentle shaking. The silica was filtered, washed with 200 mL MeOH and dried under vacuum. The UV absorbance of the hydrolysed solutions was measured at 500 nm with a Uvikon XL UV spectrophotometer (BioTek instruments, Winooski, Vermont, USA).

Analytical conditions

150 mm x 2.1 mm ID columns were slurry packed with a Haskel air driven pump (Burbank, CA, USA). 0.8 g of the derivatized silica was slurried in 7 mL MeOH/isopropanol (1/1). Packing was done with MeOH. Columns were conditioned with water at 250 µL min^{-1} until a stable base line in UV-detection and a stable back pressure were obtained. 150 mm x 3 mm ID columns were packed at Hichrom (Berkshire, England). Analyses were performed on an Agilent 1100 LC system equipped with a binary pump, autosampler, degasser and diode array detector (Agilent Technologies, Waldbronn, Germany). The system was operated with Chemstation software. Detection was performed at 254 nm with a sample rate of 80 Hz. The temperature of the chromatographic column was accurately controlled with a Polaratherm 9000 series oven (SandraSelerity Technologies, Kortrijk, Belgium). The standard samples were dissolved at 1 mg mL^{-1} in ACN and diluted with water to 50 µg mL^{-1}. The injection volume was 5 µL.

Results and Discussion

Synthesis of a PVCL based stationary phase

Initially, the same synthesis route as previously described for PNIPAAm derivatized aminopropyl silica was investigated [1]. In brief, this route consisted of the polymerisation of VCL in the presence of mercaptopropionic acid, which acts as a chain transfer agent to end the growth of the polymer chain and to provide it with a carboxylic function for anchoring to aminopropyl silica. The synthesis of PVCL was successful as could be ascertained by precipitation of the polymer in water at temperatures exceeding 35°C. Subsequently the carboxylic group at the end of the chains was activated with N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide followed by reaction with aminopropyl silica. The coupling to aminopropyl silica was, however, not successful. This is most probably due to steric hindrance of the large VCL side groups complicating the coupling reaction.

Therefore a strategy whereby the polymer is formed directly onto the aminopropyl silica was studied. This strategy has previously been described by Kanazawa et al. [22] with PNIPAAm in the presence of a cross-linking agent for making an on-silica hydrogel. The synthesis route is presented in Figure 1. 4,4'-azobis(4-cyanovaleric acid) was coupled to aminopropyl silica using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as coupling agent. Subsequently the 4,4'-azobis(4-cyanovaleric acid) modified aminopropyl silica was heated to 90°C in the presence of VCL starting the radical polymerisation. Linear VCL chains coupled to aminopropyl silica were obtained in this way.

Figure 1

The resulting silica was analysed by thermal gravimetric analysis. A weight loss of 23% was measured upon gradually heating the material up to 900°C. This carbon load exceeds the ones routinely obtained with common reversed phase materials or with PNIPAAm [1] as a stationary phase. In order to ascertain that the temperature responsive PVCL was formed on the silica, 50 mg of the latter was hydrolysed in 3 mL of a 5% NaOH solution overnight at room temperature. The UV-absorbance at

500 nm of the obtained transparent solution was measured as a function of temperature and compared to the results obtained for a solution of 3 mg/mL PVCL in water. The absorbance curves shown in Figure 2 demonstrate the temperature responsive characteristics of the polymer by an increase in absorbance when reaching the cloud point. Although both solutions show the temperature responsive characteristics the hydrolysed silica solution shows the conversion from hydrophilic to hydrophobic at a much lower cloud point compared to the PVCL solution in water. This is due to high ionic strength of the former solution which is a known parameter shifting the conversion temperature to lower values [23].

Figure 2

Chromatographic evaluation of PVCL

The thermoreponsive character of PVCL was in first instance evaluated by analysis of the steroid text mixture with pure water as mobile at 15, 25, 35 and 45°C. The column ID was 2 mm and the flow rate arbitrary set at 150 $\mu L min^{-1}$. The chromatograms are shown in Figure 3.

Figure 3

Contrary to conventional LC where retention decreases as a function of temperature (positive Van 't Hoff plots) [24], retention increases as function of temperature and negative Van 't Hoff plots are recorded. This is ascribed to an increase in hydrophobicity of the PVCL attached to the silica demonstrating the temperature responsive characteristics of the phase. Concerning resolution and efficiency, at 15°C the steroids are not separated because of the very low efficiency (1,250 plates for prednisolone) pointing out an adsorption rather than a partitioning mechanism. On the other hand, at 45°C the steroids are baseline separated and the plate number for prednisolone increased to 5,000. A similar behaviour was observed for PNIPAAm [1].

A noticeable difference between PNIPAAm [1] and PVCL is the much stronger retention of the solutes. On the one hand, this is due to the high loading of the stationary phase with PVCL, and, on

the other hand, to the increased carbon number (including the propyl from aminopropyl and the remaining part of the initiator between the PVCL chain and the silica) of the latter. The linker contains 9 carbon atoms, is not temperature responsive and will therefore remain hydrophobic at all temperatures causing severe retention for hydrophobic solutes.

As efficiencies in the temperature range 15-45°C were rather disappointing, van Deemter plots were recorded for prednisolone at 15, 45, 55 and 65°C. The H versus u plots are shown in Figure 4.

Figure 4

At 15°C, no minimum in the curve was noted because of the very slow mass transfer (C-term). At 45°C, i.e. above the LCST value where the separation mechanism is supposed to be partitioning, a normal H-u plot was obtained but H_{min} and u_{opt} are rather low, 25 µm and 0.25 mm $^{s-1}$, respectively. Efficiency increased in function of temperature H_{min} 20 at 55°C and 15 at 65°C but u_{opt} remained nearly constant. This is an unusual behaviour as for silica-based columns u_{opt} increases in function of temperature keeping the efficiency constant while for polymeric columns, both efficiency and optimal velocity increase in function of temperature [25,26]. Compared to octadecyl silica columns, a reduced plate height h (H_{min}/d_p) of 3 at 65°C is still high but not exceptional considering the low viscosity of pure water. Operating the column at 65°C and a velocity of 2.5 mm s^{-1} (flow 0.5 mL min^{-1}) gives the same efficiency as at 45°C and a velocity of 0.7 mm s^{-1} (flow 0.15 mL min^{-1}). The retention time of prednisolone in Figure 3 at 45°C of 30 min is thus reduced to less than 10 min.

Another alternative to reduce analysis times is the addition of an organic modifier. Ethanol was selected not only because it is biodegradable (green chromatography) but mainly because some of the target enzymes tolerated low concentrations of ethanol. Figure 5A shows the chromatograms for the steroid mixture at 15 and 45°C with 5% ethanol. The thermoresponsive character is still maintained while at 10% ethanol (Figure 5B) the transition from hydrophilic to hydrophobic is no longer observed. Increasing the ethanol concentration to 20% (Figure 5C) results in linear Van 't Hoff plots. Apparently, PVCL is fully soluble in this mobile phase at all temperatures and under these

conditions the material acts as a conventional stationary phase. The influence of 20% ethanol on the H-u plots is given in Figure 4 dotted lines and at 45°C the efficiency drastically increases while this is no longer the case at 65°C.

Figure 5

Because reproducibility in column manufacturing is of utmost importance in the development of robust on-line LC/bioassay methods, it was decided to outsource column packing to Hichrom that operates under ISO 9001. Selected column dimensions were 15 cm x 3 mm ID. Figure 6 shows performance chromatograms for the analysis of phenones (A) and of parabens (B) at a flow rate of 0.4 mL min^{-1} of the mobile phase water/20% ethanol at 65°C. The performance of PVCL in TR-LC with pure water at elevated temperatures for on-line LC/bioassay methods will be described elsewhere.

Figure 6

Conclusions

A PVCL-based stationary phase has been synthesised and can be used for aqueous temperature responsive LC. The expected increase in retention as a function of temperature is illustrated with a steroid standard mixture using water as mobile phase. An increase in efficiency and reduction of analysis times is noted at elevated temperatures. When adding ethanol at percentages above 10%, the temperature responsive characteristics of the phase are lost.

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Figure captions

Figure 1. Synthesis route of poly(N-vinylcaprolactam) on aminopropyl silica.

Figure 2. UV-absorbance of two PVCL solutions as a function of temperature. Open symbols: PVCL in pure water. Closed symbols: PVCL in 5 % NaOH solution and overnight hydrolysis.

Figure 3. Analysis of a mixture of six steroids with pure water at four different temperatures. Flow rate 150 µL min^{-1}, detection at 254 nm. Column: 150 mm x 2.1 mm ID PVCL.

Peaks: 1. cortisone, 2. hydrocortisone, 3. prednisolone, 4. cortexalone, 5. methylprednisolone, 6. hydrocortisone acetate.

Figure 4. Van Deemter plots at different temperatures for prednisolone. Column: 150 mm x 2.1 mm ID PVCL. Full lines: pure water. Dotted lines: 20% ethanol.

Figure 5. Analysis of a mixture of six steroids with water/ethanol at different temperatures.

A. 5% ethanol at 15 and 45°C
B. 10% ethanol at 15 and 45°C
C. 20% ethanol at 15, 25, 35 and 45°C.

Figure 6. Analysis of phenones (A) and parabens (B).

Column: 150 mm x 3 mm ID PVCL. Flow rate 0.4 mL min^{-1} water/20% ethanol at 65°C.

Peaks A. 1. acetophenone, 2. propiophenone, 3. butyrophenone, 4. valerophenone,

5. benzophenone, 6. hexanophenone, 7. Heptanophenone, 8. octanophenone.

Peaks B. 1. methylparaben, 2. ethylparaben, 3. n-propylparaben, 4. n-butylparaben

Figure 1

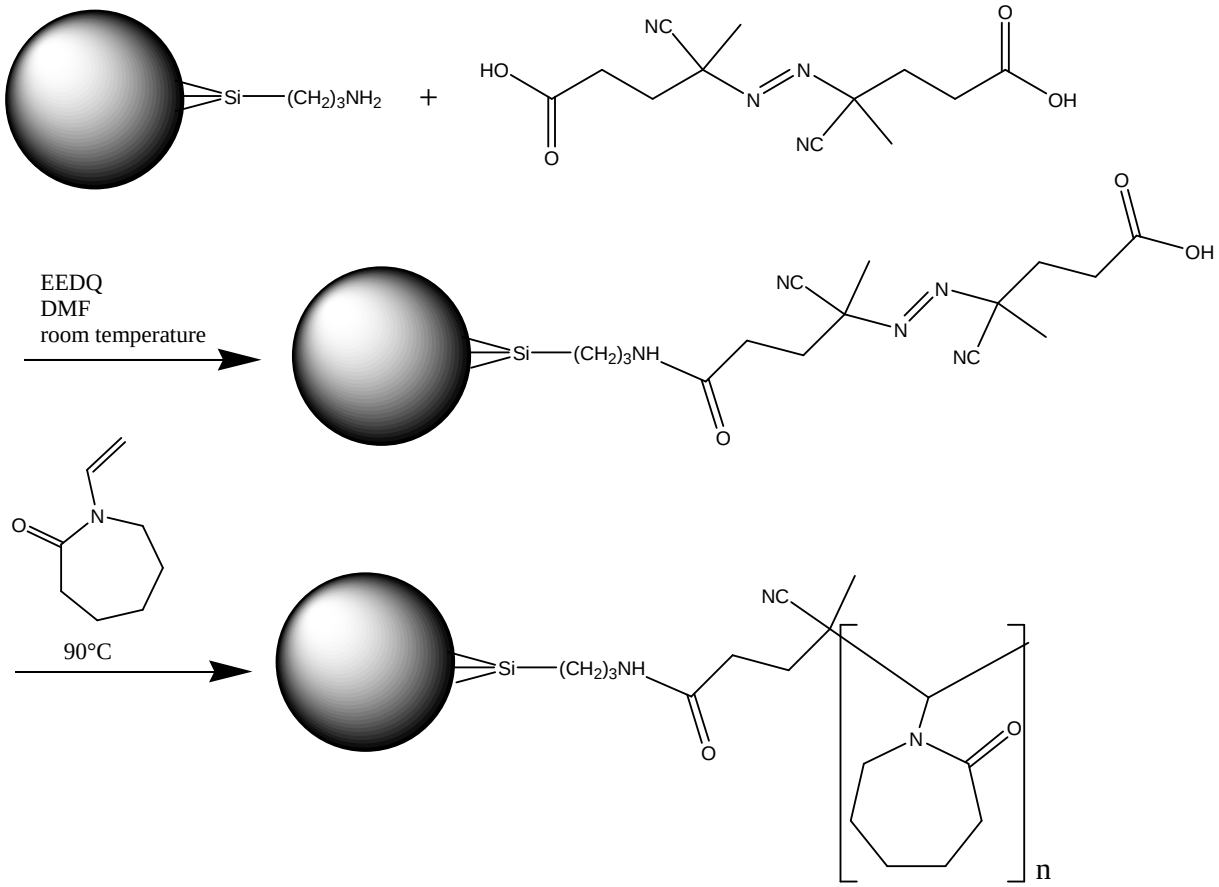
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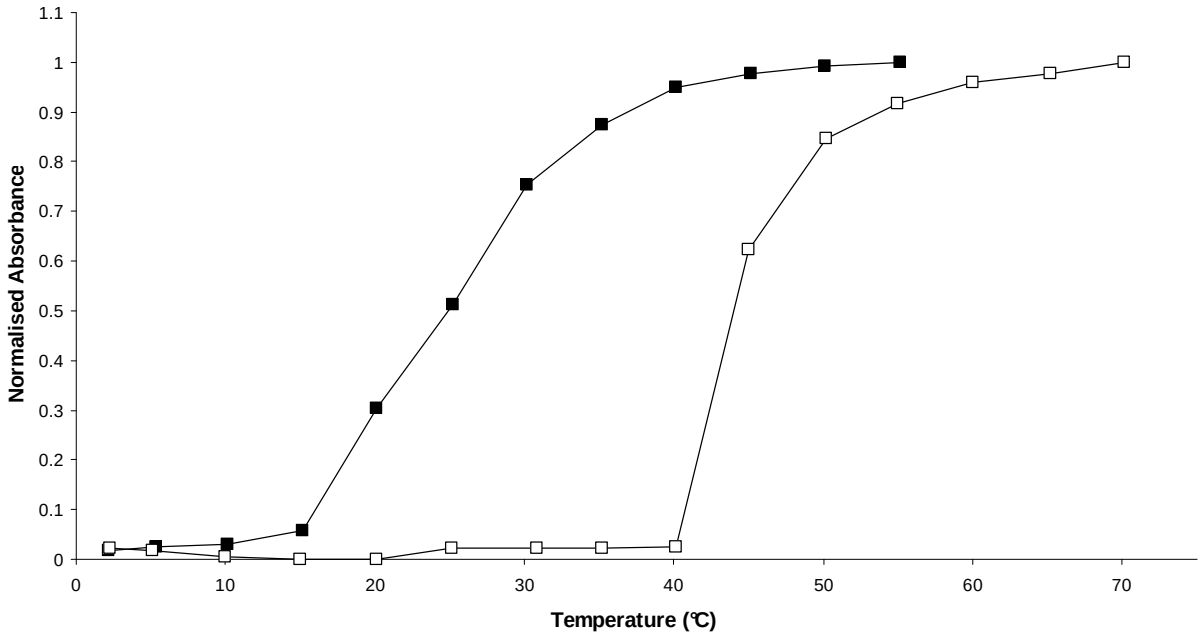
Figure 2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Temperature (℃)

Figure 3

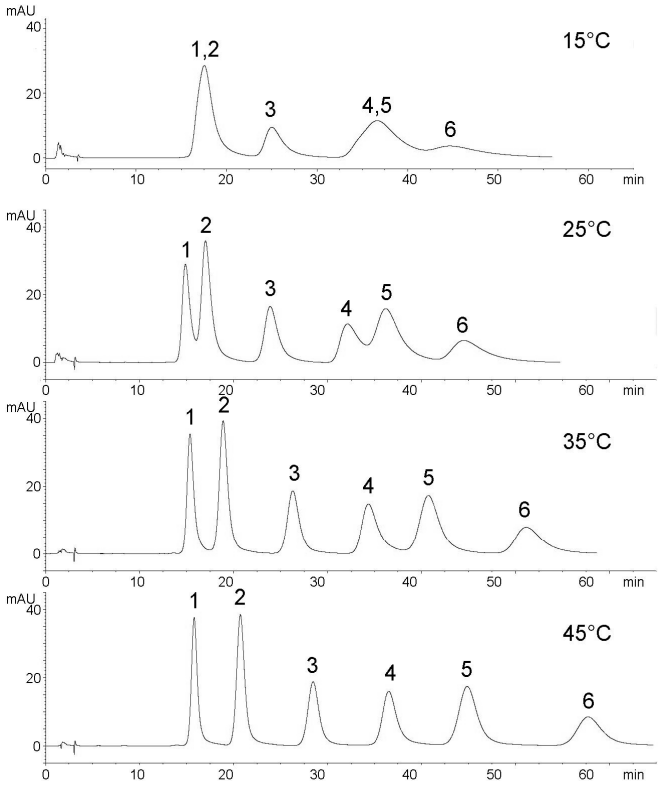
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Figure 4

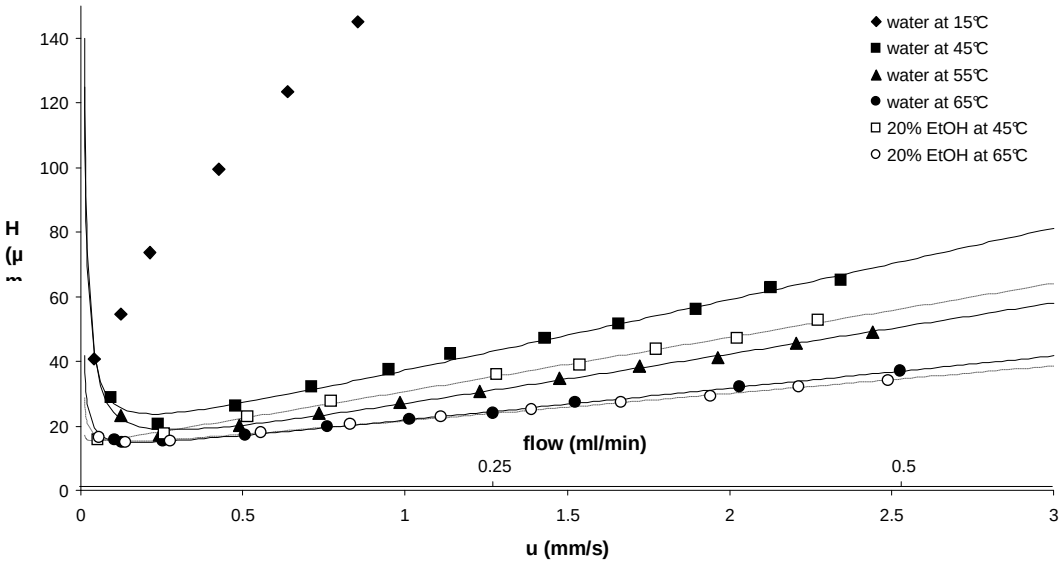
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Figure 5

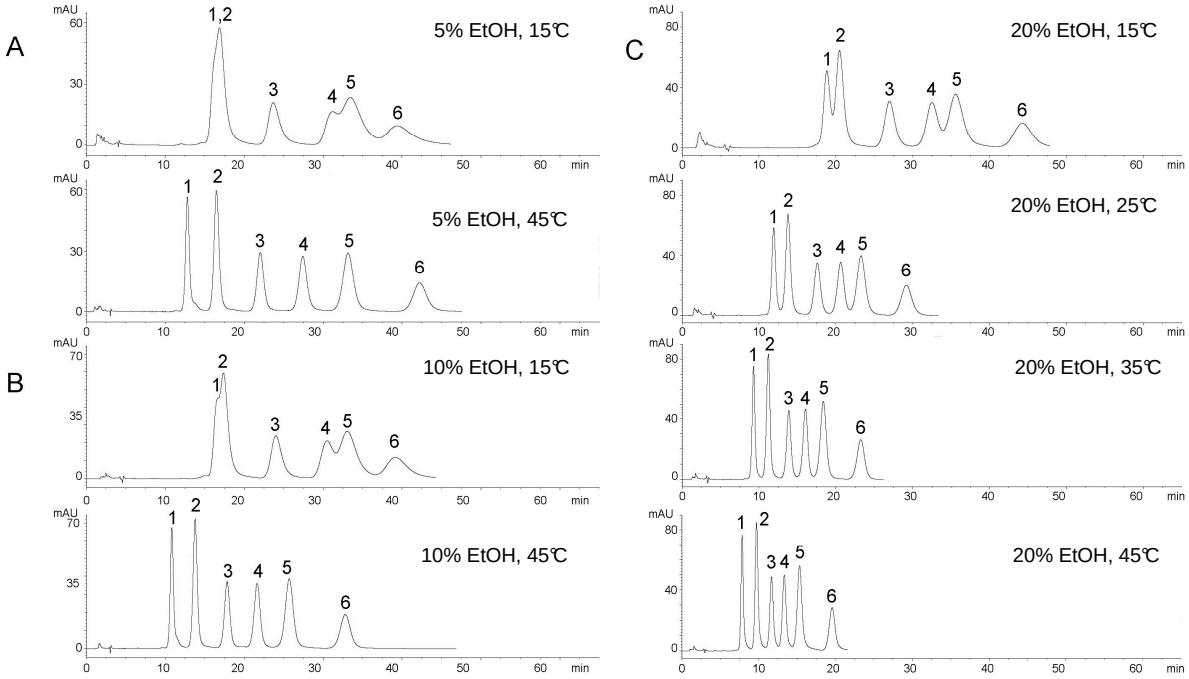
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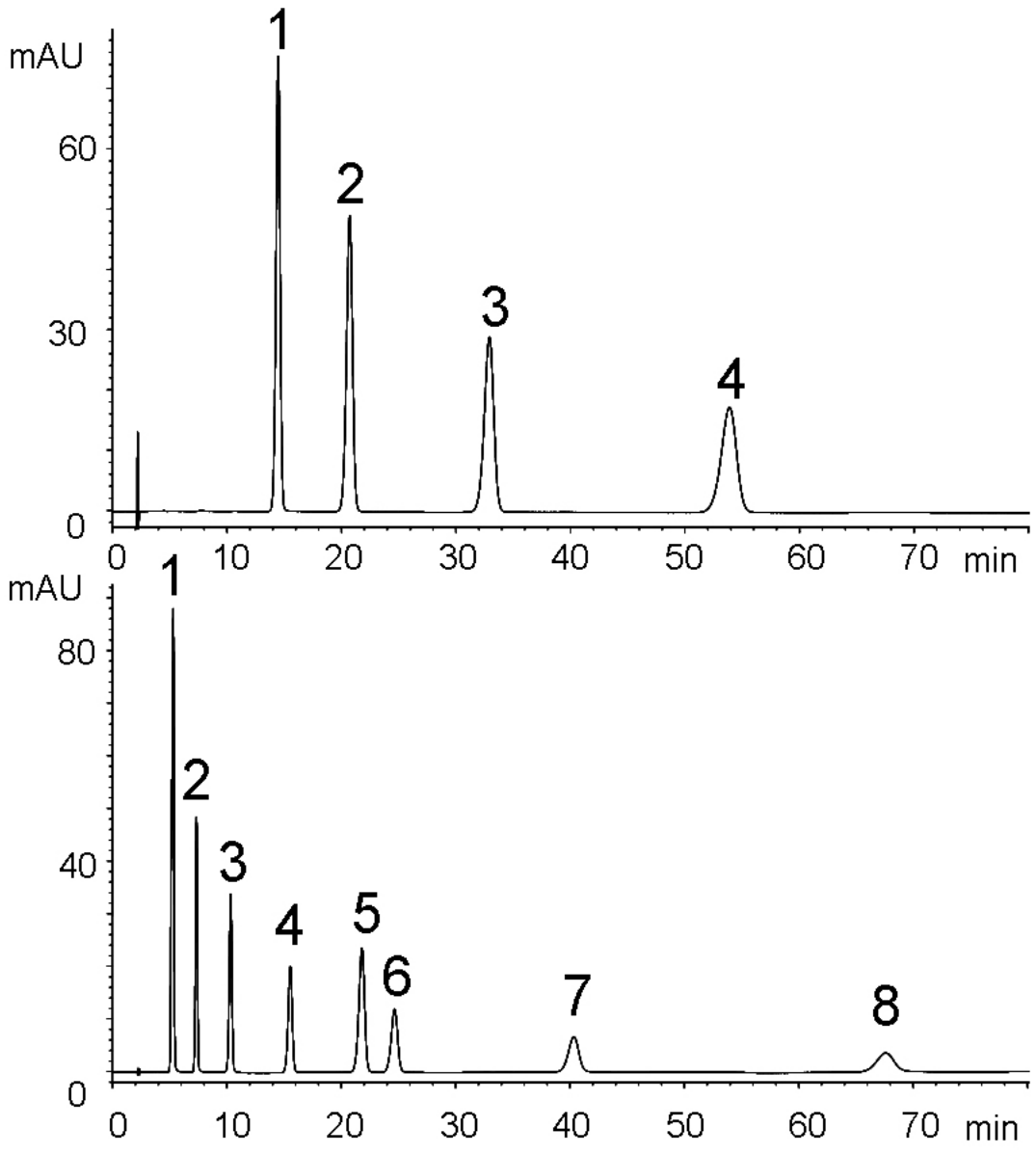
Figure 6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