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Investigation of the effects of solvent-mismatch and immiscibility in normal-phase × aqueous reversed-phase liquid chromatography

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ABSTRACT

Comprehensive two-dimensional liquid chromatography (LC × LC) is an attractive separation technique that allows achieving high peak capacities and information on chemical correlations. Unfortunately, its application in industrial practice is still not widespread due to limiting factors such as complex method development, tedious method optimization and solvent-incompatibility (such as solvent-strength mismatch or immiscibility experienced during fraction transfer).

A severe case of solvent-incompatibility is encountered in the comprehensive coupling of normal-phase LC and reversed-phase LC (NPLC × RPLC). NPLC × RPLC is considered a desirable LC × LC system, especially for the characterization of synthetic polymers, due to the high orthogonality of the two retention mechanisms. However, its experimental realization often suffers from solvent-injection effects in the RPLC dimension, such as peak-deformation, peak-splitting, or even unretained elution ("breakthrough") of sample components. Such a decrease in performance or loss of retention is highly dependent on the types of solvents used.

To explore the boundaries of solvent compatibility, we applied large-volume injections (LVI) of reference analytes (*e.g.* alkyl benzenes; ethoxylate and propoxylate polymers) dissolved in water-immiscible sample solvents, such as dichloromethane, *n*-hexane, and isooctane in fast water-based gradient RPLC separations (using methanol or acetonitrile as eluent). It was found that, when using highly aqueous initial gradient conditions, hydrophobic sample diluents were retained and eluted during the applied gradient. Depending on the relative retention of the retained diluent and the sample analytes, good chromatograms for LVI of immiscible solvents were obtained, comparable with injections under ideal conditions. The conclusions from injection experiments in aqueous RPLC were verified by coupling an NPLC system with a gradient from isooctane to tetrahydrofuran and an RPLC system with a gradient from water to acetonitrile in an online comprehensive NPLC × RPLC separation of a mixture of propoxylate polymers. The separation provided separation of the polymers based on their number of hydroxyl end-groups (NPLC) and oligomer chain-length (RPLC), without suffering from significant band-broadening effects due to solvent-mismatch upon injection in the second-dimension RPLC system.

1. Introduction

Liquid chromatography (LC) is an important analytical technique for the characterization of many chemicals in a wide variety of application areas, such as pharmaceuticals [1], polymer chemistry [2], life science [3], and food products [4]. Although one-dimensional LC (1D-LC) is a very powerful technique, the maximum attainable efficiency and peak capacity is limited and typically ranges from about 100 in a few minutes to a few hundred in a few hours [5]. Therefore, 1D-LC is often unable to separate complex mixtures or chemically similar species, which are difficult to resolve. In such cases, two-dimensional liquid chromatography (2D-LC) may be advantageous [5]. Furthermore, 2D-LC often provides structured chromatograms, allowing for convenient detection and identification of unknown species.

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In comprehensive two-dimensional liquid chromatography (LC × LC), the total effluent of a first-dimension (^{1}D) LC separation is fractionated by means of a modulator valve and all fractions are subjected to a second-dimension (^{2}D) separation. Very high peak capacities can be obtained, since the peak capacities of the two individual separations may be multiplied, while the total analysis time is only slightly longer than the time required to complete the ^{1}D separation. To make full use of the potentially high peak capacities of LC × LC, the two separation dimensions must be independent of each other, *i.e.* separate the sample based on different ('orthogonal') retention mechanisms [6].

Despite the demonstrated strength of LC × LC to resolve very complex mixtures, its widespread application in industry is hampered by various factors. Examples include complex method development and instrumentation, long time required for method optimization and incompatibility issues encountered between the two orthogonal separation dimensions [7,8]. Solvent incompatibility is often encountered in LC × LC because the ^{1}D effluent becomes the injection solvent of the ^{2}D separation in relatively large quantities. As a result, the ^{2}D separation is often compromised in terms of peak shapes and peak capacity. Examples of such incompatibility issues include viscous fingering when viscosity differences exist between the ^{1}D and ^{2}D mobile phases, resulting in flow instability and, hence, distorted chromatographic peaks [9]. Furthermore, incompatibility issues may arise from immiscibility of two mobile-phase systems encountered when, for instance, normal-phase LC is coupled with reversed-phase LC (NPLC × RPLC), where non-polar organic solvents are used in the ^{1}D system, which cannot be freely introduced in an aqueous ^{2}D RPLC separation. In addition to the immiscibility issue, severe mismatch of elution strength is observed between the strong injection solvent from the ^{1}D (high in organic content) and the ^{2}D mobile phase. As a result, the analytes may be poorly retained on the stationary phase, resulting in deformed or split peaks or in unretained elution of (part of) the sample. This latter phenomenon is known as 'breakthrough' and is often observed in polymer analysis when using a comprehensive combination of size-exclusion chromatography (SEC) and RPLC (SEC × RPLC) [10]. Breakthrough may also occur in gradient elution 1D-LC of polymers, when the analytes require a strong organic solvent to be solubilized, while maintaining chromatographic integrity simultaneously requires the injection solvent to act as a weak eluent at the initial conditions of the chromatographic analysis. This combination is difficult to achieve [11,12].

Improvements of the applicability of LC × LC have largely focused on instrumental development to overcome the mobile-phase incompatibility between two separation dimensions. Instead of 'passively' storing relatively large quantities of ^{1}D effluent in sample loops installed on the modulator, which causes problems upon injection onto the ^{2}D column, recent modulation techniques 'actively' circumvent incompatibility issues. Common examples include stationary-phase-assisted modulation and active-solvent modulation [10,13,14]. Furthermore, hardware solutions were developed, such as in-column focusing [15], vacuum-membrane-evaporation modulation [16], temperature-responsive stationary phases [17] and thermal modulation [18].

Although these developments are extremely useful, most of the techniques are in early stages of development and not widely applied, due to increased hardware complexity, limited availability, costs, and difficulty of operation. This is supported by the fact that approximately 75% of the strategies currently applied in LC × LC involve loop-based (*i.e.* passive) modulation [7]. NPLC × RPLC is deemed to be an incompatible combination, due to the immiscibility of the eluents used in the two separation dimensions. Therefore, examples of the successful application of NPLC × RPLC in recent literature are very rare [8]. This is unfortunate, as NPLC × RPLC is one of the most orthogonal LC × LC combinations and worth exploring for compounds of medium-to-low polarity. Long before the introduction of active modulation techniques, good results were obtained with NPLC × RPLC systems using loop-based approaches. Such an analytical strategy was applied for the analysis of carotenoids in red mamey fruit [19], red chili peppers [20] and red orange essential oils [21]. In these studies, various method parameters contributed to successful NPLC × RPLC coupling. Firstly, high percentages of isopropanol in the ^{2}D eluent were used to create a phase system that was miscible with the ^{1}D effluent that was rich in *n*-hexane (> 75% v/v). Furthermore, narrow-bore ^{1}D columns (1.0 mm I.D.) were used, operated at low flow rates to reduce the modulation volumes, while wide-bore ^{2}D columns (4.6 mm I.D.) were operated at high flow rates to diminish injection effects. Finally, the large carbon-backbone structures of the analyzed carotenoids implied strong interaction with C18 stationary phases, allowing non-aqueous RPLC to be used in the second dimension.

Aqueous ^{2}D RPLC has also been successfully coupled with ^{1}D NPLC, which is more difficult from a solvent-immiscibility perspective. Polyphenols and (furano-)coumarins were successfully characterized in citrus-oil extracts [22–25] and the separation of various analytes in pharmaceutical and reference mixtures was described [24,25]. Again, narrow-bore ^{1}D columns (1.0 mm I.D.) were used, while wide-bore ^{2}D columns (4.6 mm I.D.), operated at high flow rates (4 – 5 mL/min), were applied to minimize injection effects. Francois et al. [24,25] used relatively large modulation volumes, injecting 30 to 40 µL fractions that contained at least 92% *n*-hexane onto ^{2}D C_{18} columns, run in gradient-elution mode with an initial mobile-phase concentration of 0 or 35% ACN. Remarkably, good ^{2}D separations were obtained and no significant peak distortions or breakthrough of analytes were observed. Unfortunately, for lemon-oil compounds eluting at high ethyl-acetate concentrations from the ^{1}D column, ^{2}D peak distortion and band broadening were observed. However, an interesting observation was made in off-line RPLC experiments, in which the injection solvent consisted of *n*-hexane and ethyl acetate and the ratio of these two solvents was varied. At higher *n*-hexane concentrations, where the injection solvent was immiscible with the RPLC mobile phase, the focusing of the solutes actually improved (data not shown in the study). Although these observations are counterintuitive, given the consensus that water-immiscible solvents result in broad or distorted peaks in (partly aqueous) RPLC, the results might indicate injection phenomena that are not yet fully understood and that may aid in the successful realization of NPLC × RPLC.

While solvent compatibility in LC × LC has not been exhaustively explored, large-volume injection (LVI) of water-immiscible sample diluents in one-dimensional RPLC has been previously studied by various authors and the approach appears to be feasible [26–31]. Such LVI strategies have been applied for impurity profiling in pharmaceutical and bio-medical analysis, in cases in which solubility of the target-analytes in a water-miscible solvent was limited and a high sensitivity was demanded. This necessitated direct injection of samples dissolved in a hydrophobic organic solvent, often under LVI conditions to obtain high sensitivity. Sample diluents with good chromatographic results for aqueous RPLC separations included alkanes (*n*-hexane, *n*-heptane, isooctane, *n*-decane, *n*-dodecane), aliphatic alcohols (1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol), isopropyl acetate, and methyl isobutyl ketone. Using C_{18} columns with relatively large internal diameters (150 × 4.6 mm I.D., 5 µm particle diameter, d_p) and mobile phases containing 78 to 95% water, injection volumes of pure organic diluent (*n*-hexane, *n*-heptane, isooctane) could range up to 500 µL, while maintaining good peak shapes [29]. Such findings are supported by other studies that showed successful chromatography of the analytes, provided that the hydrophobic diluent eluted after the analyte of interest, *i.e.* that the sample diluent was more

strongly retained than the analyte. If the organic diluent eluted before the analyte of interest, peak broadening and distortion were observed [30].

Various explanations have been proposed as to how pure organic diluents with high elution strengths could successfully be used in RPLC analysis. One of the hypothesis implied that, under aqueous conditions, the organic diluent was retained by the stationary phase, where it would compete with the analytes for adsorption sites on the stationary phase [26]. If the sample diluent were to have greater affinity for the stationary phase than the analyte, peak shapes would not be affected. This would also imply that, upon retention of the sample diluent on the stationary phase, part of the stationary surface would become unavailable for the analytes. This hypothesis is supported by a linear decrease in retention of the analyte with increasing injection volume [26,27]. Another hypothesis was based on reversed-phase supported liquid-liquid extraction [28]. Upon injection of a hydrophobic diluent under aqueous mobile phase conditions, the diluent was thought to be retained on the head of the column. When the mobile phase subsequently entered the column, it was thought to create a liquid film of diluent immobilized on the stationary phase. The analytes would then be extracted from the diluent film to the mobile phase by liquid-liquid extraction and separated by conventional liquid chromatography on the uncovered stationary phase further down the column.

Although the documented studies of large-volume injections in diluent that were not miscible with the RPLC mobile phase provided interesting insights, the results cannot be directly translated to NPLC × RPLC. In these studies, isocratic separations were usually conducted using relatively long, wide-bore columns with large column volumes (typically 150 × 4.6 mm I.D., d_p = 5 µm). In contrast, RPLC applied as a ^{2}D separation in a LC × LC system increasingly uses low-volume, narrow-bore columns, packed with sub-2-µm particles (for example, 50 × 2.1 mm, d_p = 1.7 µm, $V_m \approx$ 104 µL) for fast separation under gradient-elution conditions. Due to the stricter constraints on ^{2}D RPLC separations, the effects of the large-volume injection of immiscible diluents could be more significant in LC × LC systems.

In this study, we investigated the solvent effects observed for different weakly and strongly retained analytes under extreme solvent-mismatch and non-miscibility conditions encountered in NPLC × RPLC. A wide variety of pure organic solvents commonly used in NPLC were studied as sample diluents in aqueous RPLC gradient-elution separations. Injection volumes and column dimensions were in line with the current state-of-the-art of LC × LC. To study the retention and elution of diluents and analytes independently, ultraviolet/visible spectroscopy (UV/Vis) and evaporative-light-scattering detection (ELSD) were used in series. This allowed decoupling of the diluent and analyte signals. Furthermore, retention factors and loadability were determined for various diluents. To demonstrate the possibility of performing successful LC × LC separation under extreme solvent-mismatch conditions, we aimed to develop an NPLC × RPLC-UV/Vis-ELSD separation capable of resolving propoxylates based on their terminal hydroxyl composition and oligomer chain length. Simultaneously, we aimed to elucidate the elution behavior of the ^{1}D NPLC effluent in the ^{2}D RPLC separation.

2. Experimental

2.1. Chemicals

The solvents used in this study included methanol (MeOH, ULC/MS grade), acetonitrile (ACN, LC-MS grade), 2-propanol (isopropanol, IPA, LC/MS grade) and tetrahydrofuran (THF, unstabilized LC-MS grade) obtained from Biosolve B.V. (Valkenswaard, The Netherlands). *n*-Hexane (> 99.5%, HiPerSolv grade), dichloromethane (DCM, >99.8%, HiPerSolv grade), chloroform ($CHCl_3$, >99.8%, HiPerSolv grade) and 2,2,4-trimethylpentane (isooctane, > 99.5%, HiperSolv grade) were obtained from VWR International B.V. (Leuven, Belgium). Deionized water (Arium 611UV, Sartorius, Göttingen, Germany; R = 18.2 MΩ cm) was used for aqueous solutions. To study the separation performance under large-volume-injection (LVI) conditions, butanol-initiated propoxylates, glycerol-initiated ethoxylates, and a 'reference mixture' consisting of benzyl alcohol, benzaldehyde, and 2-nitrotoluene were utilized. For NPLC × RPLC experiments a mixture of butanol-initiated propoxylate, polypropylene glycol and glycerol-initiated propoxylate was used. The polymeric materials were kindly supplied by Dow Benelux B.V. (Terneuzen, The Netherlands). The individual chemicals used for the reference mixture were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.2. Instrumentation

One-dimensional LC experiments were performed using a Waters Acquity UPLC system (Waters, Milford, MA, USA). The system comprised of a binary solvent manager, sample manager, column manager, photo-diode-array (PDA) detector equipped with an analytical flow cell (V_{det} = 500 nL) and an evaporative light-scattering detector (ELSD). Two-dimensional LC experiments were performed using an Agilent 1290 Infinity 2D-LC system (Agilent Technologies, Waldbronn, Germany). The system comprised of a high-speed binary pump (G7120A) and a binary pump (G7112B) for solvent delivery. Other components included a multicolumn thermostat (G7116A), autosampler (G71676A), a diode-array detector (G4212A) equipped with an Agilent Max-Light cartridge flow-cell (G4212–60,008, 10 mm path length, V_{det} = 1.0 µL) and an ELSD (G7102A). An Agilent 2D-LC ASM valve (G4243A) connected with two distinct multiple heart-cutting valves equipped with 40-µL loops were used for modulation and operated in comprehensive 2D-LC mode (ASM feature was not used throughout the study). Headspace gas chromatography – mass spectrometry (HS-GC/MS) experiments were conducted using an Agilent GC system (7890A) hyphenated with a mass selective detector (Agilent 5975C) employed with the Perkin Elmer Turbo Matrix 40 Trap headspace sampling module.

One-dimensional RPLC experiments were performed using a Waters Acquity BEH phenyl-hexyl column (50 × 2.1 mm, 1.7 µm particle size) (Waters). For NPLC × RPLC experiments, an Ascentis Express OH5 (50 × 2.1 mm I.D., 2.7 µm particle size) column (Sigma-Aldrich) was used in the first-dimension, while the aforementioned RPLC column was used as second-dimension column. GC experiments were performed using a J&W VF-1701 ms GC column (30 m × 0.32 mm I.D., 1 µm film thickness).

2.3. Analytical conditions

For one-dimensional RPLC analysis with LVI, samples were prepared at predefined concentrations in the different organic diluents that are listed in Table 1, together with their corresponding properties. For the propoxylates and ethoxylates, sample solutions of 0.25 mg/mL were prepared separately in the corresponding organic diluents. For the standard mixture, benzyl alcohol, benzaldehyde, and 2-nitrotoluene were combined at a concentration of 0.005 mg/mL for each analyte in varying organic diluents. Full-loop injections of 20-µL of the studied analytes in the eight different organic diluents were performed. Separations were performed at 30 °C at a flow rate of 0.5 mL/min using the following gradient from water (mobile phase A) to acetonitrile (mobile phase B): 0.0–0.2–3.0–4.0–4.01–5.5 min, 5–5–95–95–5–5%B. For the separation of ethoxylates, the same separations using LVI of chlorinated solvents were repeated with MeOH (mobile phase B) as or-

Table 1
solvent properties of studied organic diluents.

Solvent	Viscosity (cP)[a]	Solvent strength (silica) [a]	Polarity index [a]	Water Miscibility	Partition coefficient ($Log P_{OW}$)[b]
Water	1.00		10.2		
Methanol	0.55	0.70	5.1	Yes	−0.74
Isopropanol	2.40	0.60	3.9	Yes	0.05
Acetonitrile	0.38	0.52	5.8	Yes	−0.34
Tetrahydrofuran	0.55	0.53	4.0	Yes	0.46
Dichloromethane	0.44	0.30	3.1	No	1.25
Chloroform	0.57	0.26	4.1	No	1.97
n-Hexane	0.31	0.00	0.1	No	4.00
Isooctane	0.50	0.00		No	4.60

[a] values reproduced from [32].
[b] Octanol-water partition coefficients, values reproduced from [33] and safety data sheets of corresponding compounds.

ganic modifier instead of acetonitrile. To obtain 'reference' chromatograms (*i.e.* chromatograms obtained using low-volume injections with suitable solvents for retention), 5-µL injections were used for the propoxylates and ethoxylates at a concentration of 1 mg/mL in MeOH, while a 2-µL injection was used for the NPLC standard mixture at a concentration of 0.05 mg/mL for each analyte in ACN.

To determine retention factors of DCM, THF, *n*-hexane and isooctane under isocratic conditions at varying percentages of organic modifier (ACN), 1-µL partial-loop injections with needle overfill (PLNO) of pure organic diluent were performed under isocratic conditions at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% ACN using a flow rate of 0.5 mL/min and thermostatting at 30 °C.

To determine column properties (pore volume and interstitial volume), PMMA (1.0 MDa) was used as fully excluded polymer prepared at a concentration of 0.1 mg/mL in THF, while uracil dissolved in 60:40 water:ACN at a concentration of 0.1 mg/mL was used to determine the void volume of the column. For uracil, the column was operated under isocratic conditions of 40% ACN at a flow rate of 0.5 mL/min, while for PMMA, 100% THF was used as eluent to prevent precipitation or retention of the polymer.

To study the retention behavior of organic diluents under overloading conditions, 20-, 50-, 75- and 100-µL injections of THF, DCM, *n*-hexane, or isooctane were performed using a flow rate of 0.5 mL/min with the following gradient program: 0.0–5.0–5.01–7.0–7.01–9.0 min 5–5–95–95–5–5%B.

For all one-dimensional RPLC analyses, UV/Vis and ELS detection were performed in series. The delay volume between the UV/Vis and ELSD detectors was 22.5 µL, corresponding to 0.045 min at a flow rate of 0.5 mL/min. The ELSD chromatograms were corrected for the inter-detector delay time. UV/Vis detection was performed at 20 Hz with acquisition channels of 210, 225, 254, 270, and 300 nm, at 4.8 nm resolution. ELSD was performed using a 20-Hz acquisition rate and a set gain factor of 500, 20 psi (300 kPa) N_2 gas pressure, nebulizer set to cooling, and the drift tube operated at 60 °C.

For NPLC × RPLC analysis, a mixture of butanol-initiated propoxylate, polypropylene glycol and glycerol-initiated propoxylate was prepared in a 1:1:1 ratio at a concentration of 5 mg/mL each in 35% THF / 65% isooctane (v/v). 5 µL of the sample were injected onto the ^{1}D column thermostatted at 30 °C and operated with a flow rate of 0.017 mL/min. The following gradient program was applied using 35% THF in isooctane (v/v) (mobile phase A) and THF (mobile phase B): 0.0–5.0–30.0–40.0–40.01–80.0 min, 0–0–95–95–5–5%B. The modulation time was set to 1.2 min, corresponding to a modulation volume of 20.4 µL. The ^{2}D RPLC separation was thermostatted at 60 °C and operated at a flow rate of 1.2 mL/min. The following gradient from water (mobile phase A) to ACN (mobile phase B) was used: 0.0–0.01–1.0–1.01–1.2 min, 5–45–90–5–5%B. The ELSD was operated using an acquisition rate of 80 Hz, 60 °C evaporator temperature, 60 °C nebulizer temperature, and a gas flow of 1.6 SLM. UV/Vis detection was performed at a sampling rate of 40 Hz with the acquisition of 210-, 230-, and 254-nm wavelength channels.

To confirm the elution of the ^{1}D solvents as function of the ^{2}D separation as depicted by UV/Vis, the ^{2}D effluent was fractionated and subsequently analyzed by HS-GC/MS. During a NPLC × RPLC run as described above, nine fractions over a single ^{2}D run were collected in sampling vials and subjected to HS-GC/MS analysis te reveal the presence of isooctane and/or THF in each fraction. For HS sampling, the vials were heated to 90 °C for a duration of 15 min. Subsequently, 1 µL was injected in split-mode at a split ratio of 30:1 operated at 200 °C. For the GC separation, the flow rate was set at 1.7 mL/min using helium as carrier gas. The initial oven temperature of −20 °C was held for 2 min, followed by a 10 °C/min ramp to 240 °C and held at the final temperature for 10 min. The MSD transfer line was set at 280 °C and MS detection was performed at a mass range from 20 – 550 *m/z* using 70 eV electron ionization. Extracted ion chromatograms (EIC) were constructed for THF and Isooctane using the ions at *m/z* 72.0 and 57.0, respectively. Integration of the EICs provided the relative abundance of THF and isooctane in the analyzed fractions.

2.4. Data acquisition and treatment

One-dimensional LC-UV/ELSD chromatograms were acquired using Empower 3 software suite (Waters, Milford, MA, USA). The LC × LC-UV/ELSD system was controlled using OpenLab CDS Chemstation version C.01.07SR2. The data was exported as space-separated .CSV files and processed using MatLab 2018a (Mathworks, Woodshole, MA, USA), LC × LC chromatograms were constructed using in-house written code. HS-GC/MS instrument control was performed using OpenLab version C01.07SR3 and processed using MassHunter Qualitative Analysis software version B.07.00. Chromatograms of organic diluents under overloading conditions (20–100 µL injections) were smoothed in MatLab 2018a (Mathworks, Woodshole, MA, USA) using Savitzky-Golay filtering with a second-order polynomial and a frame length of 101.

3. Results and discussion

3.1. 1D-RPLC with large-volume injection under extreme-solvent-mismatch conditions

Based on the polarity index and solvent-strength of common organic solvents as provided in Table 1, the coupling of NPLC × RPLC is troublesome, as solvent-strength mismatch and solvent-miscibility issues are often inevitable. Interestingly, in our experiments we observed that solvent-mismatch conditions may not significantly influence the chromatographic separation in aqueous RPLC if both the sample diluent and the analytes are retained under the initial mobile-phase conditions and if the ana-

lytes elute prior to the diluent. We systematically studied this type of behavior under conditions applicable to aqueous RPLC as ^{2}D separation in an NPLC × RPLC system, using large-volume injections (LVI) of various (often immiscible) solvents with high elution strengths. The studied sample diluents are listed in Table 1, where we provide the viscosities of common pure HPLC solvents, together with their respective solvent strengths, polarity indices, water-(im)miscibility and octanol-water partition coefficients ($LogP_{ow}$) [32,33]. To realize LVI, 20-µL volumes of the analyte solutions in pure organic solvent were injected on a Waters Acquity Phenylhexyl column (50 × 2.1 mm, 1.7 µm d_p), corresponding to approximately 20% of the column void volume (104 µL). This column was chosen because overloading conditions were easily created (due to its low volume) and the impact of strong diluents would be more severe as compared to conventional C_{18} columns due to the reduced retention. To study the solvent effects with highly aqueous starting conditions, the initial mobile phase composition was 95% water. This was maintained for 0.2 min, followed by a linear gradient to 95% organic modifier in 2.8 min to separate the compounds of interest. To deconvolute the signals of the diluent and the analyte, UV/Vis and ELS detection were used in series. In such a set-up, the elution of the diluent could be traced by UV/Vis separately from the signal obtained for the analyte acquired by ELSD. Propoxylate oligomers were used (~1 kDa), which yield a series of defined peaks with ELSD, whilst showing no UV/Vis absorbance. Furthermore, a mixture consisting of small reference molecules (benzyl alcohol, benzaldehyde and 2-nitrotoluene) soluble in most of the organic solvents was used in this study. These analytes are often encountered in NPLC and RPLC and are less retained than the propoxylates, representing a more difficult separation case as the separation of 'early eluters' is often compromised under solvent-mismatch conditions [34].

The LVI (20 µL) chromatograms of propoxylates and a standard mixture with eight different organic solvents as diluent are shown in Figs. 1 and 2, respectively, and summarized in Table S1 and S2 of the Supplementary Information (retention time and peak-widths). Using small-volume injections of more concentrated samples, 'reference' chromatograms were obtained for the studied samples (Figs. 1a & 2a). In Fig. 1a, the ELSD signal depicts the separation of propoxylates according to oligomer chain-length (with longer chains eluting at longer retention times, elution window about 2.2 to 3.2 min). Under similar conditions, three distinct peaks are obtained for the standard mixture with the compounds eluting according to polarity (Fig. 2a, elution window about 1 to 2 min). Below we summarize our observations from the separations of the two samples starting from the water-soluble solvents, followed by the non-water soluble solvents.

For LVI of propoxylates with water-miscible solvents, acceptable chromatograms are obtained for MeOH, ACN and IPA as diluents compared to the reference conditions. Although the peak-width at half-height ($W_{0.5}$) of the most abundant propoxylate signal increases slightly, being 0.035, 0.045 and 0.028 min for MeOH, ACN and IPA, respectively (as shown in Table S1 of the Supplementary Information) compared to 0.023 min for the reference conditions, the diluents are weak enough to promote retention (Figs. 1b, 1c, 1d). No breakthrough was observed, although it is believed that all three diluents elute at the void volume of the column, as confirmed for MeOH through the UV/Vis diluent signal (no diluent signals were measured for ACN and IPA due to UV/Vis transparency). On the other hand, for THF, which is a stronger solvent than the aforementioned diluents, breakthrough of the propoxylates was observed with a large portion eluting around t_0 and a smaller portion eluting in the expected retention range with acceptable peak widths ($W_{0.5}$ of 0.035 min). The breakthrough signal clearly overlapped with the diluent peak detected at t_0.

Compared to the propoxylates, the results for the small-molecule mixture with decreased retention were more significantly affected by the injection solvent. The separations were severely distorted when using LVI with MeOH, ACN, IPA, and THF as diluents, as shown in Figs. 2b, 2c, 2d and 2e, respectively. Both breakthrough and severe peak fronting were observed for all three sample components, as well as increased $W_{0.5}$ for especially the first two eluting compounds as shown in Table S2 of the Supplementary Information. This underlines the relative importance of the relationship between the solvent-strength of the diluent and the retention of the studied analytes. If the diluent plug is swept through the column while its solvent-strength prohibits retention on the stationary phase, simultaneous elution of a (large) fraction of the analytes can be expected.

Interestingly for dichloromethane and chloroform, different retention behaviors were observed. Although DCM and $CHCl_3$ are stronger diluents than MeOH, ACN, IPA, and THF under RPLC conditions, no breakthrough was observed for the propoxylates and standard mixture as shown in Figs. 1f, 1g, 2f, and 2g (no analyte signal present at t_0). By examination of the UV/Vis signals, it becomes clear that the elution of these diluents is delayed, *i.e.* they are retained by the stationary phase upon injection (Figs. 1f and 1g UV/Vis signal). Upon elution of the diluent by the programmed gradient, a fraction of the propoxylate sample is also eluted, which overlaps with the diluent signal. Relative to DCM, $CHCl_3$ desorbs a greater fraction of the propoxylates within the elution zone of the diluent, which can be explained by the increased solvent strength of $CHCl_3$ compared to DCM. The resolution of the remaining solutes, which elute in the expected retention window (2.2–3.2 min) is negatively affected by both diluents. Unfortunately for the small-molecule mixture with LVI of DCM and $CHCl_3$, the diluent elutes in the same elution window as the analytes, as can be seen in Figs. 2f and 2g. Since the UV/Vis signal is saturated across the wavelength range where the analytes absorb, chromatographic profiles of the studied analytes could not be obtained.

For *n*-hexane and isooctane, which are even stronger diluents in RPLC than the previously discussed injection solvents, very good chromatograms were obtained (in comparison with the previous discussed diluents) for both the propoxylates and the standard mixture, as shown in Figs. 1h & 1i and 2 h & 2i, respectively. Although *n*-hexane and isooctane do not absorb in the UV/Vis range, their elution could be traced due to their immiscibility with the aqueous mobile phase and accompanying disturbance in the UV/Vis flow cell. This was also noticed in other studies [28,35]. To confirm the late elution of these solvents, off-line fractionation of the RPLC effluent resulting from the injection of pure diluent was performed. The fractions were analyzed by HS-GC/MS (see Section 3.4 below). As can be seen for *n*-hexane and isooctane in Figs. 1h and 1i, the diluents were strongly retained and eluted after the elution-zone of the analytes. As a result, the propoxylate sample and the standard mixture could be separated by the applied gradient, with similar $W_{0.5}$ as under the reference injection conditions. These results are better than those obtained with the other studied organic diluents. A decrease in retention times was observed for both the propoxylates and the standard compounds in comparison with the reference conditions. The most abundant propoxylate oligomer eluted at 2.71 min under reference conditions, while it eluted at 2.57 min with *n*-hexane as diluent (−0.14 min) and at 2.54 min with isooctane (−0.17 min), respectively. A similar trend was observed for the standard compounds. The retention time of the three analytes decreased slightly (−0.18, −0.19, −0.11 min for benzyl alcohol, benzaldehyde and 2-nitrotoluene, respectively) compared to the reference conditions. This is in agreement with previous studies [26,27], where increased injection volumes of the non-miscible solvent resulted in a decrease of analyte retention. This was ascribed to a decrease in

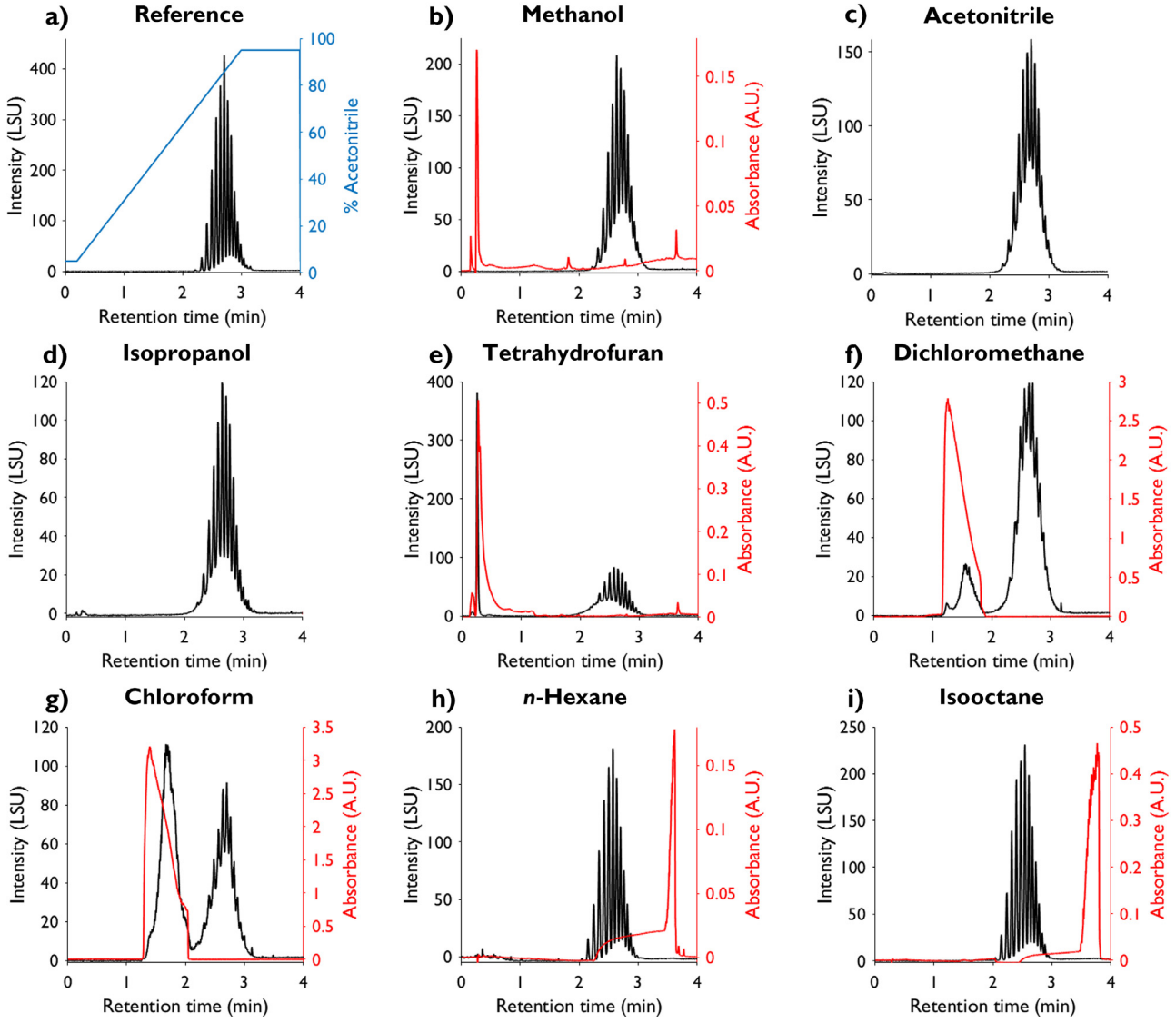

Fig. 1. Separation of propoxylates (black, ELSD) and organic diluent (red, UV/Vis) under (a) reference conditions (with programmed gradient shown in blue) and LVI using (b) MeOH, (c) ACN, (d) IPA, (e) THF, (f) DCM, (g) $CHCl_3$, (h) *n*-hexane, and (i) isooctane as organic diluent. For ACN and IPA, no clear diluent signal was obtained and, therefore, not shown. For detailed chromatographic conditions, see the Experimental Section.

the available stationary-phase surface upon retention of the diluent. Benzaldehyde and, especially, 2-nitrotoluene are broadened in comparison with the reference chromatograms for both *n*-hexane and isooctane as diluent based on the $W_{0.5}$ values supplied in Table S2 of the Supporting Information. It is not yet fully understood why this occurs. A possible explanation may be the poor partitioning of benzaldehyde and, to a greater extent, 2-nitrotoluene from the hydrophobic diluent immobilized on the stationary phase (functioning as a – temporary – liquid stationary phase) to the mobile phase, assuming the analytes are retained by the hydrophobic diluent. Another explanation could be a dual-partitioning effect of the analytes between the stationary phase and the mobile phase and between the immobilized hydrophobic diluent and the mobile phase.

The location of the elution zone of the retained diluent with respect to the elution-zone of the analytes is an important indicator for successful LVI under extreme-solvent-mismatch conditions. If the diluent eluted at t_0 and was a sufficiently strong solvent for the analytes, breakthrough was observed. On the other hand, if the diluent eluted after the analytes, successful chromatographic separations were obtained without interference of the sample diluent. When the diluent was retained at the initial mobile-phase composition but eluted before or during the elution window of the analytes, non-optimal results were obtained, as observed for the propoxylates. Since the UV/Vis absorbance of DCM and $CHCl_3$ obscured the signal of the standard mixture, the effect of diluent elution on the analyte chromatograms could not be studied.

3.2. Importance of organic modifier for chlorinated diluents

To deconvolute the diluent signals of DCM and $CHCl_3$ and the signals of analytes with small retention factors, the experiments with DCM and $CHCl_3$ were repeated using a more hydrophilic class of polymers, *viz.* ethoxylates. The greater hydrophilicity of the sample results in shorter elution times during the RPLC run (1.0–1.5 min), similar to those of the small-molecule standards. In comparison with those standards, the ethoxylates did not provided a UV–Vis response, whilst clear signals in ELSD could be obtained, successfully deconvoluting sample and diluent signals. Moreover, the greater hydrophilicity of the sample allowed separations to be

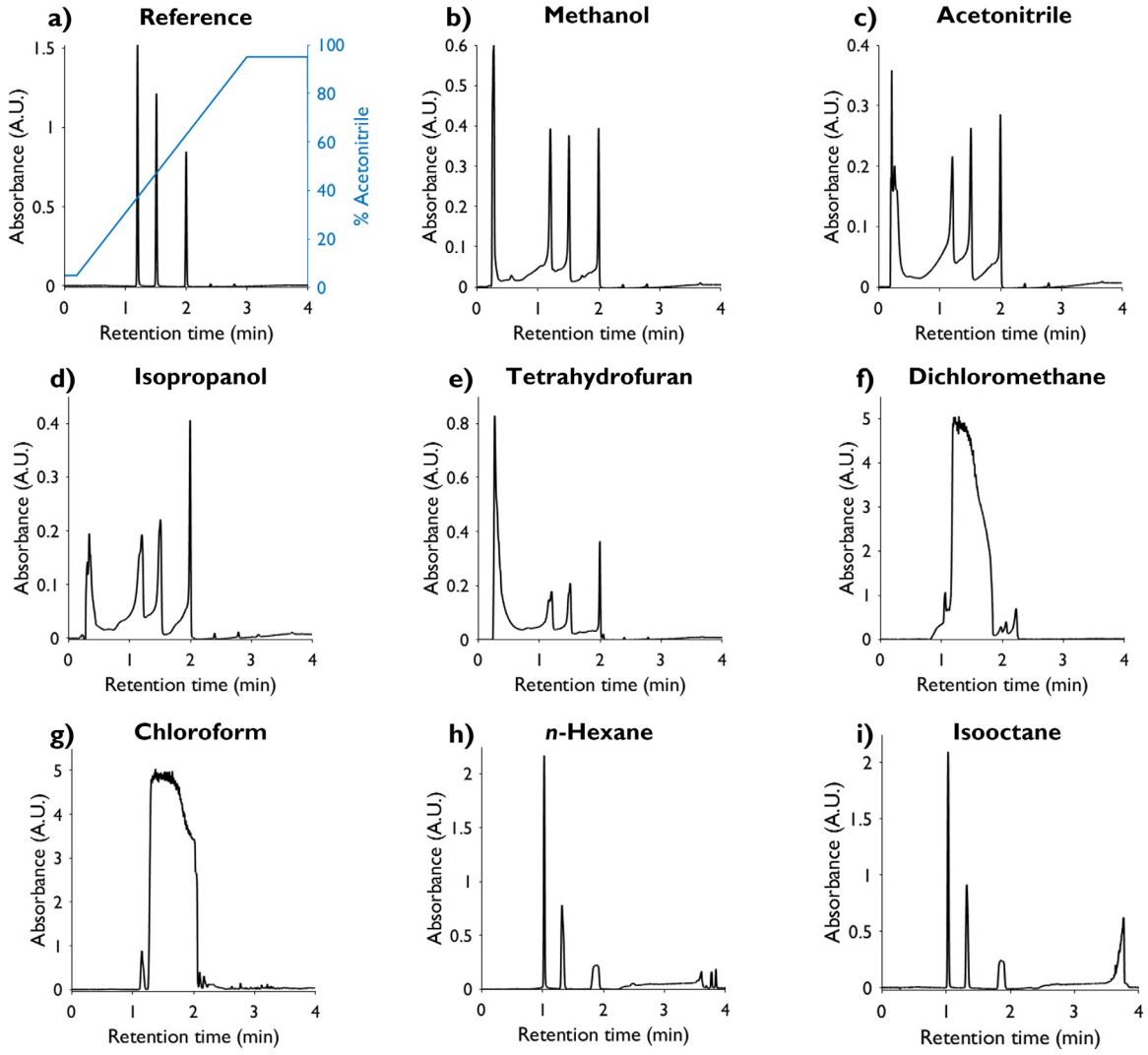

Fig. 2. separation of a standard mixture (consisting of benzylalcohol, benzaldehyde and 2-nitrotoluene) under (a) reference conditions (with programmed gradient shown in blue) and LVI using (b) MeOH, (c) ACN, (d) IPA, (e) THF, (f) DCM, (g) $CHCl_3$, (h) *n*-hexane, and (i) isooctane as organic diluent. For detailed chromatographic conditions, see the Experimental Section.

performed both with ACN and MeOH as organic modifiers. Separations with MeOH were explored since the solubility of chlorinated solvents in water-MeOH mixtures is known to be greater than in water-ACN mixtures.

Using ACN as modifier, the ethoxylates eluted as a single sharp peak at the onset of the elution of either DCM or $CHCl_3$ (Figs. 3b and 3c, respectively). The peak was much sharper than that obtained under reference conditions (Fig. 3a). Surprisingly, the use of MeOH for gradient elution led to improved separations, as shown in Figs. 3e and 3f. A gradual elution of the diluents was observed when using MeOH as modifier, which could be explained by the increasing solubility of chlorinated solvents in ternary systems with decreasing amounts of water and increasing amounts of MeOH during the gradient. This resulted in good separation of the ethoxylates with comparable $W_{0.5}$ to the reference conditions (Table S3 of the Supplementary Information) up until the end of the elution window of the diluent, at which point the higher-MW ethoxylates were compressed into a single peak. At the end of the diluent elution, the concentration of the chlorinated solvent may be high enough to promote desorption of the remaining analytes, which could explain the observed chromatograms. An alternative explanation may be that the chlorinated solvent acts as a dynamic stationary phase, increasing the retention of the ethoxylates as long as it is present.

3.3. Retention of organic diluents under volume-overloading conditions

As became apparent in the gradient-elution experiments, organic diluents were retained to different extents on the stationary phase under highly aqueous conditions, after which desorption was achieved using the applied gradient. The relatively polar, water-miscible solvents showed little or no retention on the stationary phase, and, hence, often led to breakthrough of the analytes studied. The hydrophobic solvents, which were not miscible with water, provided interesting opportunities as injection solvents depending on the relative elution times of the analytes and the diluent. The retention behavior of organic diluents at various mobile-phase compositions was studied under isocratic conditions using small-volume injections (1 µL). As is apparent from Fig. 4, THF showed very little retention at 2% ACN ($k \approx 1$), with retention quickly diminishing to $k \approx 0$ with increasing ACN content. The retention factor of DCM in highly aqueous mobile phases was higher than that of THF. Up to about 10% ACN k was about 5, after which it decreased with increasing ACN concentration. An exponential in-

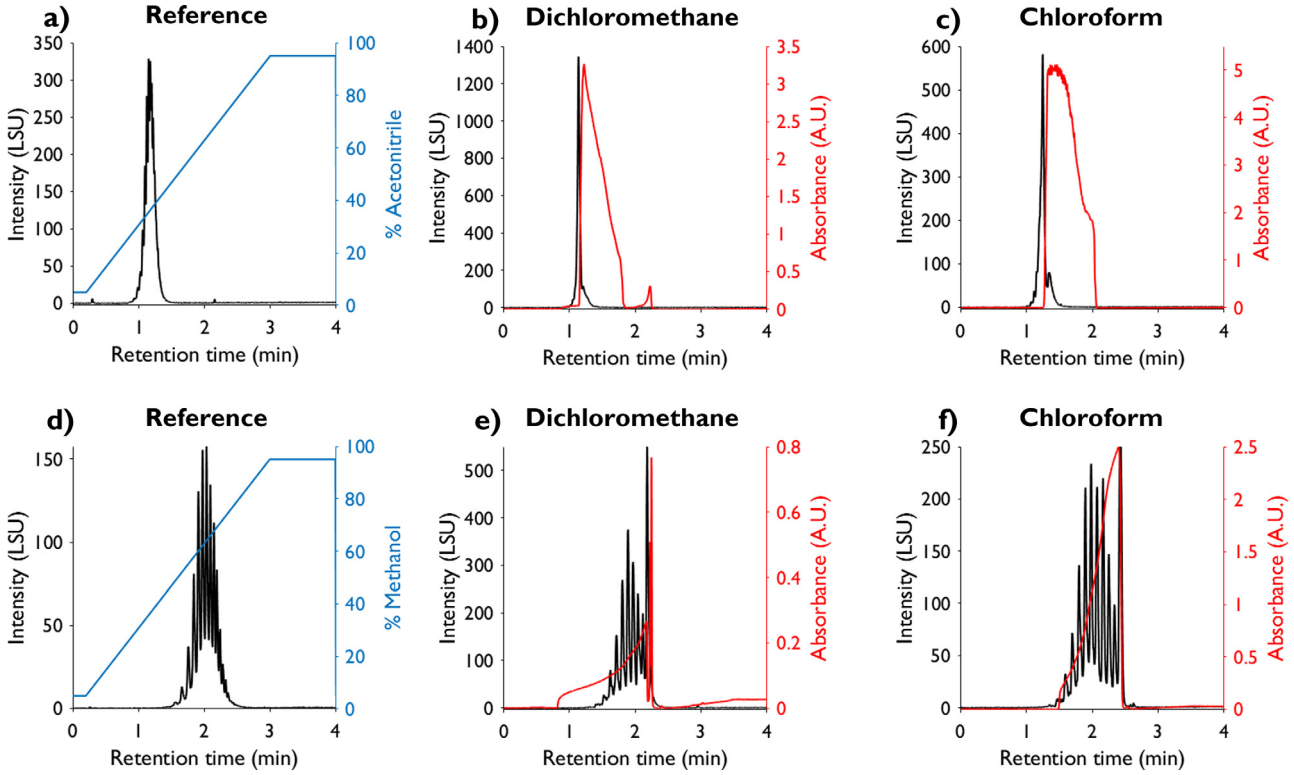

Fig. 3. separation of ethoxylates using (a-c) ACN as organic modifier or (d-f) MeOH as organic modifier (% B, programmed gradient shown in blue) when using LVI of DCM and $CHCl_3$. For detailed chromatographic conditions, see the Experimental Section.

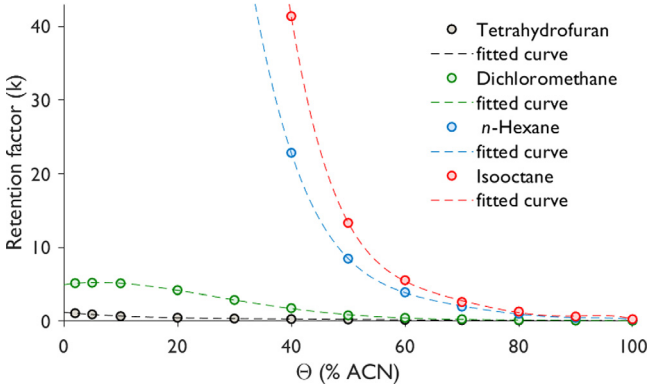

Fig. 4. Measured retention factor *versus* organic-modifier concentration (isocratic conditions) for THF (black), DCM (green), *n*-hexane (blue), and isooctane (red). For detailed chromatographic conditions, see the Experimental Section.

crease in retention factors was observed when moving from high to low percentages of ACN for both *n*-hexane and isooctane, with isooctane being more strongly retained. Clearly, these hydrophobic solvents were fully retained under the highly aqueous conditions typically encountered at the start of aqueous RPLC gradients. The retention curves shown in Fig. 4 allows us to establish ranges for the initial and final percentages of modifier in the mobile phase that allow retention and elution of the diluent, respectively.

To study the volume-overloading behavior of the studied diluents under highly aqueous mobile-phase conditions, the injection volume was increased from 20 µL up to 100 µL. According to the theory of total pore blocking [35], the hydrophobic diluent replaces the bulk mobile phase present in the intra-particle pores upon injection and remains inside the pores due to hydrophobic interactions with the stationary phase layer and the immiscibility with the surrounding hydrophilic mobile phase. If this theory is true, the column could retain hydrophobic diluents up to the pore volume of the column; if the injection volume exceeds the pore volume, the excess diluent that remains in the interstitial volume is believed to be displaced by the flowing mobile phase and accompanying hydrodynamic force from the pump and should appear as 'breakthrough' in the chromatograms. To verify this theory, the pore volume of the column was estimated and a set of organic diluents were used at injection volumes below, equal and larger than the pore volume.

From the elution times of a t_0 marker and a fully excluded polymer sample column characteristics were determined. A total column volume of 104 µL and a pore volume of 49.5 µL were determined (interstitial volume 54.5 µL; ratio of pore volume to column volume 48%). To study the retention when approaching or exceeding the total pore volume, LVI of 20, 50, 75, and 100 µL of organic diluent were performed. Isocratic conditions were maintained at 5% ACN for 5 min to evaluate the 'breakthrough' of diluent under solvent overloading conditions, followed by a step gradient to 95% ACN and another isocratic hold for 2 min to completely elute the diluent from the column. As can be seen in Fig. 5a for the water-soluble THF, the diluent elutes at t_0 for all injection volumes. For DCM with LVI of 20 µL as shown in Fig. 5b, hardly any breakthrough was observed during the isocratic hold at 5% ACN. The 'breakthrough' increased with increasing injection volume, with most of the diluent continuously eluting during the isocratic hold at 5% ACN. DCM was found to not be fully retained under LVI conditions, possibly due to the low but significant solubility of DCM in water. These results may explain the poor results described for DCM in previous section(s). For both *n*-hexane and isooctane, however (Fig. 5c and 5d), it can be seen that both 20 µL and 50 µL injections lead to full retention during the 5% ACN isocratic hold, with the diluent only being eluted from the column during the step gradient to 95% ACN. The LVI of 50 µL roughly

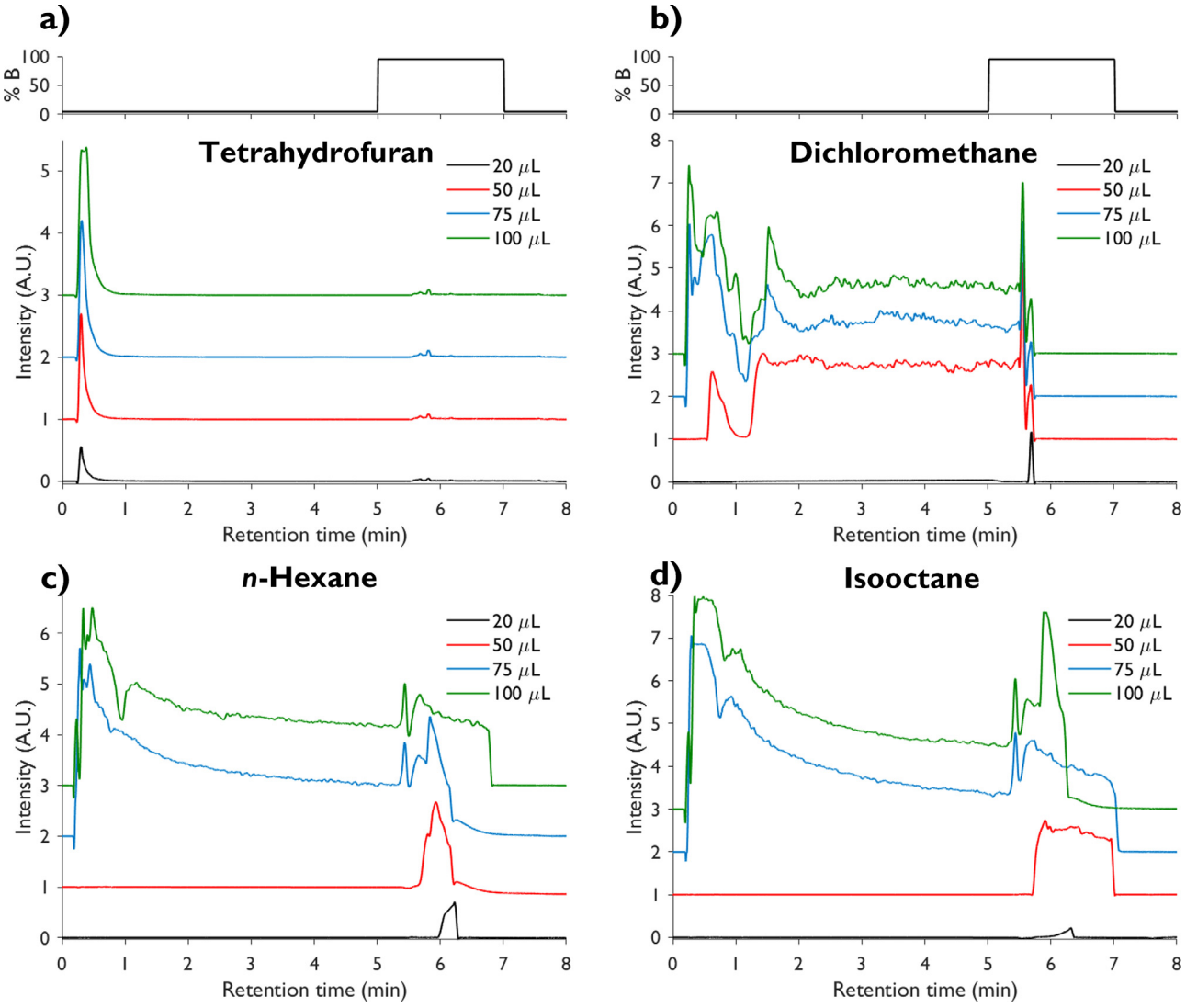

Fig. 5. 20 µL (black), 50 µL (red), 75 µL (blue) and 100 µL (green) LVI of a) THF, b) DCM, c) *n*-hexane and d) isooctane. Injections were performed under isocratic-hold conditions of 5% ACN for 5 min, followed by a step-gradient to 95% ACN kept for 2 min, as shown by the subplots at the top of the Figure. For detailed chromatographic conditions, see the Experimental Section.

equals the pore volume of the column. When increasing the LVI to 75 or 100 µL, volumes that exceeded the estimated pore volume, 'breakthrough' of the non-miscible diluent was observed with continuous elution during the isocratic hold. Such results are helpful to establish the upper boundaries in LC × LC with respect to injection volume *versus* breakthrough.

3.4. NPLCxRPLC-UV/ELSD

As shown in the previous section, relatively high volumes of *n*-hexane and isooctane can be retained upon injection at highly aqueous conditions. The studied injection volumes with respect to the total column volume closely resemble conditions encountered in LC × LC separations. Since propoxylates could be separated very well in RPLC using LVI of *n*-hexane and isooctane, an one-dimensional NPLC method was established, capable of resolving propoxylate samples according to the number of terminal hydroxyl groups, independent of oligomer chain-length. The latter could subsequently be determined using RPLC as the second-dimension separation. The conditions for this NPLC × RPLC separation closely resemble the studied conditions as discussed above. A gradient using high content of isooctane was used in the ^{1}D NPLC separation, from which fractions (modulation volumes) of 20.4 µL were introduced onto the same RPLC column as used for the one-dimensional experiments (50 × 2.1 mm, 1.7 µm) at highly-aqueous initial mobile phase conditions (5% ACN).

As can be seen from the NPLC × RPLC-ELSD chromatogram in Fig. 6a, three different propoxylate distributions, corresponding with mono-ol, diol and triol species were separated in the first dimension, with an increasing number of hydroxyl end-groups resulting in longer ^{1}D retention times. The different functionalities were separated in the ^{2}D system according to increasing oligomer chain-length with increasing elution times. No peak distortion or breakthrough was observed. From the NPLC × RPLC-UV chromatogram shown in Fig. 6b, it was determined that the THF eluted at t_0, while the isooctane eluted later in the chromatogram (between about 58 and 70 s), after the elution window of the analytes. The retention of the non-miscible diluent and subsequent desorption during the ^{2}D gradient is a highly repeatable cycle, confirmed by both the ELSD and UV LC × LC chromatograms. To confirm the composition of the diluent zones as indicated in the NPLC × RPLC-UV chromatogram (Fig. 6b), fractions of 2D cycles were collected off-line (as illustrated in Fig. 6c) and analysed using HS-GC/MS for the presence of THF and isooctane. The re-

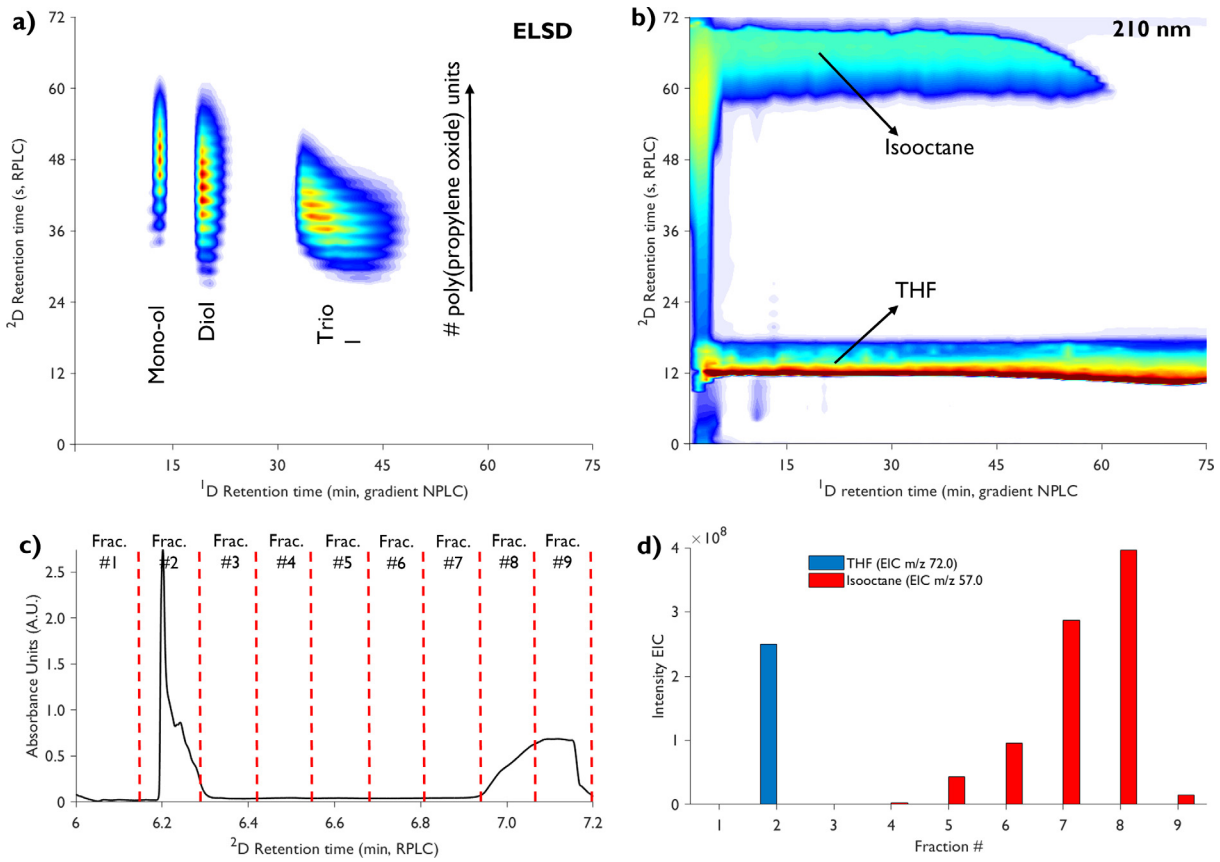

Fig. 6. separation of a) propoxylate mixture according to the number of hydroxyl end-groups and oligomer chain-length using NPLC × RPLC and the b) elution zones of organic solvents eluting from the ^{1}D column visualized by UV/Vis detection. To confirm the composition of the elution zones of the ^{1}D effluent, the ^{2}D effluent was c) fractionated off-line and d) analyzed using HS-GC/MS. For detailed chromatographic conditions, see the Experimental Section.

sults (Fig. 6d) confirmed that THF eluted early from the ^{2}D column (fraction 2), while isooctane eluted towards the end of the ^{2}D gradient (fractions 5–8), completely in line with the one-dimensional RPLC experiments descibed in previous paragraphs.

3.5. General discussion and applicability of NPLC × RPLC

Although solvent-strength mismatch, immiscibility and viscous fingering encountered in loop-based NPLC × RPLC were a concern prior to the NPLC × RPLC method development, we have shown in this study that the presence of such phenomena does not necessarily result in poor chromatography. With proper LC × LC method optimization, good results can be obtained irrespective of whether viscous-fingering, solvent-strength mismatch or immiscibility occur. The NPLC × RPLC-UV separation showed repeatable retention and elution of isooctane in each modulation, which resulted in successful oligomer speciation in the ^{2}D RPLC separation, as revealed by the LC × LC-ELSD chromatogram. This is in accordance with the one-dimensional RPLC LVI experiments, where *n*-hexane and isooctane proved to be favorable diluents. In accordance with previous studies [26,27], reduced retention factors were observed for LVI with *n*-hexane and isooctane diluents, as compared to injection under reference conditions. This can be explained by a reduction in the number of available adsorption sites or stationary-phase surface area for the analyte molecules due to retention of the diluent plug. This implies that the analyte molecules are extracted by the mobile phase from the diluent upon injection onto the stationary phase, as proposed by *Lazar et al.* [28]. The diluent plug may act as a locally immobilized liquid stationary phase, which allows partitioning of the sample analytes. It's difficult to fully understand the observed injection phenomena from experimental data alone, because a ternary-phase system should be considered in the presence of a stationary phase. Molecular-dynamics simulations may be applied to provide better insights in the discussed phenomena.

In general, in NPLC × RPLC the content of the polar water-miscible desorption-promoting solvent in the ^{1}D NPLC system should be kept as low as possible, as such solvents are more likely to cause breakthrough and peak distortions in the second dimension. Depending on the relative retention of the studied analytes and the solvent-strength of the polar NPLC modifier, its volume fraction in each modulation can be increased. On this basis the modulation volume may be increased to allow faster LC × LC analysis. In the current study, the modulation volume was limited to about 20 µL at 65/35% isooctane/THF (v/v), due to pressure restrictions in the ^{2}D system. As the ^{2}D separation was performed using a narrow-bore column (2.1 mm ID) with sub-2 µm particles, operated at a high flow rate of 1.2 mL/min using 95% water as initial conditions, relatively high back-pressures were observed. In addition, pressure spikes were typically observed upon injection of the modulated fractions. These approached the pressure limit of the ^{2}D pump (about 120 MPa). The spikes were thought to be caused by the sudden introduction of a large amount of isooctane and the accompanying interfacial tension of the isooctane–water system. To avoid problems due to pressure-spikes, the operating ^{2}D back-pressure was decreased by lowering the flow-rate to 1.2 mL/min. This was a limiting factor for the LC × LC method and might be the case for NPLC × RPLC in general when water-immiscible solvents are used.

4. Conclusion

We have investigated solvent effects observed in the loop-based comprehensive coupling of normal-phase LC (NPLC) and reversed-phase LC (RPLC). Upon large-volume injection (LVI) of organic diluents at highly aqueous initial mobile phase conditions in the RPLC separation, immiscible diluents were found to be retained on the stationary phase and eluted from the column using the applied gradient. Depending on the relative elution windows of the retained diluent and the analytes, good chromatographic results were achieved for *n*-hexane and isooctane – the strongest RPLC diluents in this study. Large volumes of these diluents could be retained on the column.

The process of diluent retention and subsequent elution using fast gradient RPLC separations proved to be highly repeatable in an LC × LC setting. Successful NPLC × RPLC separations of a mixture of propoxylates could be achieved by separating the polymeric distributions based on the number of hydroxyl end-groups in the first dimension and on oligomer chain length in the second dimension. The NPLC × RPLC-UV chromatogram revealed distinct ^{2}D RPLC elution zones of the THF and isooctane solvents applied in the first dimension. The polar, water-miscible THF eluted close to the void volume, while the hydrophobic water-immiscible *n*-hexane eluted at the end of the gradient.

Although solvent-strength mismatch, immiscibility and/or viscous fingering are encountered in loop-based NPLC × RPLC, this does not mean that such LC × LC methods cannot be developed. Proper optimization of the NPLC × RPLC method may result in high-quality results. The key findings for successful loop-based NPLC × RPLC found in this work are: *i*) analytes of interest must be retained by the second dimension, *ii*) (a large portion of) the diluent (first-dimension effluent) must be retained by the second-dimension and *iii*) the retained diluent must elute after the analytes of interest from the second dimension. The developed NPLC × RPLC may be applied to a variety of samples, including polymers and small-molecule analytes, in research or industrial settings.

Declaration of Competing Interest

Statement: "The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper."

CRediT authorship contribution statement

Gino Groeneveld: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization, Project administration. **Melissa N. Dunkle:** Conceptualization, Resources, Investigation, Writing – review & editing, Supervision. **Matthias Pursch:** Conceptualization, Writing – review & editing, Supervision. **Edwin P.C. Mes:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Peter J. Schoenmakers:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Andrea F.G. Gargano:** Conceptualization, Methodology, Writing – review & editing, Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.462818.

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