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High-resolution polymer high performance liquid chromatography: Application of a saw tooth gradient for the separation of various polymers

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ABSTRACT

Currently, a lot of research effort in polymer analysis by liquid chromatographic techniques, including size exclusion chromatography (SEC), polymer HPLC or liquid chromatography at critical conditions, is done aiming to improve separation performance. In this study, novel gradient protocols were investigated primarily based on gradient polymer elution chromatography (GPEC). Starting with linear gradients and stepwise gradients a new periodic saw tooth gradient profile was developed and optimized. Optimum settings for the saw tooth gradient design were evaluated by design of experiments (DoE) based on Taguchi's methodology for various types of stationary phases. The gain of peak resolution was dependent on the effective gradient step height. The optimized protocol enabled high-resolution polymer HPLC (HRP-HPLC) separations with common HPLC instruments. The quality of separation was evaluated by heart-cut fraction collection of HRP-HPLC and subsequent determination of the individual fractions by SEC or MALDI-ToF mass spectrometry. Finally, different types of polymers, such as PVC, PDMS, PMMA, or PPG, were studied with the new method and a universal applicability was shown.

1. Introduction

The investigation of polymers, with heterogeneous composition in more than one distribution property, is a challenging task. Applying various types of liquid chromatographic techniques, separations according to molar mass, chemical composition or polymeric architecture can be achieved. In recent years, different approaches for the separation of complex polymeric materials were used, e.g. isocratic or gradient SEC as well as liquid chromatography at critical conditions (LCCC) [1,2]. The connection between these different modes of polymer separation techniques can be explained by thermodynamic treatment [3–6]: Under ideal SEC conditions the separation depends on entropy changes only, while under ideal liquid adsorption chromatography (LAC) conditions the separation only depends on enthalpy changes. In SEC, polymers are separated due to their different hydrodynamic volumes in a solvent and, thus, a molecular mass distribution can be obtained. Therefore, no interaction between the polymer and the stationary phase should occur or virtually be minimized. In LAC, polymer analysis is mainly determined by interactions between analyte, mobile and stationary phase. Therefore, a variety of different parameters must be adjusted. If enthalpic and entropic changes equalize each other and thus the change of free Gibbs energy becomes zero critical conditions are realized. At critical conditions, LCCC for isocratic elution or critical point of adsorption (CPA) for gradient elution, molar mass does not contribute to retention volume, enabling separations solely based on differences in chemical composition. Compared to LCCC, applying gradient elution for separation of polymers at critical conditions provides a separation system which is not terminated by the pore size of the stationary phase [1,4,6]. However, each method has its own advantages and disadvantages. The constraints of SEC include for example secondary enthalpy driven interactions or indirect molar mass determination by measuring the hydrodynamic volume of the polymer. Furthermore, depending on the pore volume and molecular weight of investigated samples, low separation performance with broad peaks may occur [6]. Nevertheless, SEC provides an enhanced resolution especially in the high molecular mass range of polymers, compared to gradient LAC where at the critical point of adsorption no separation due to molar mass differences is possible [1].

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As well as in SEC, liquid chromatography at critical conditions, shows disadvantages in reproducibility, susceptibility to fluctuations, sample recovery or overall application to minor changes of the sample matrices. Beside this, in polymer HPLC, optimizing the parameters of measurement is often difficult concerning the choice of proper stationary and mobile phase combinations, e.g. adsorption promoting and desorption promoting solvents or a retention promoting column for the investigated polymer. Furthermore, the diversity of various separation parameters makes method development challenging and time consuming [6–9].

Compared to HPLC of small molecules, polymer HPLC especially differs in terms of small diffusion coefficients of the constituents in solution and a different retention mechanism of polymers on the stationary phase. A further difference between macromolecules and small molecules is the objective of the chromatographic separation, whereby in HPLC of small molecules, the exact identification and quantification is in focus and in polymer HPLC, the fractionation of macromolecules based on various polymer characteristics, e.g. molar mass distribution, size in diluted solution, chemical distribution, or chain structure, prevails. In addition, polymer retention lasts as long as at least one repetition unit of the polymer is adsorbed to the stationary phase. Unlike in HPLC of small molecules, polymers must be dissolved in very strong solvents, e.g. THF or toluene, considering reversed phase polymer HPLC. Consequently, solubility effects become important in addition to adsorption and partition phenomena. The injection of the dissolved polymers at the (usually strong adsorption promoting) initial conditions in gradient polymer elution chromatography (GPEC) result in precipitation or strong adsorption of the analytes on the head of the column [1,10–12]. With increasing amounts of desorption promoting solvent, the (homo-)polymers elute in reversed elution order compared to SEC, from low to high molar masses, at least as long as the critical point of adsorption is not reached. Therefore, it is not only sufficient that a solvent is a strong solvent for the investigated polymer. Additionally, the separation system (mobile and stationary phase) must provide desorption promoting characteristics for the used strong polymer solvent. Apart from molar mass differences, chemical functionalities cause an additional separation, especially dominating in the low molecular mass region [3,13]. The separation occurs predominantly according to adsorption effects to the stationary phase and precipitation effects depending only on the solubility in the mobile phase. In addition to LAC, Glöckner et al [7] termed separations without adsorption effects to the stationary phase high performance precipitation liquid chromatography (HPPLC). Staal [14] showed the similarities between cloud point determination by turbidimetric titration and the precipitation- / redissolution processes in the different steps of the chromatographic separation for reversed phase systems. In the first step, the polymer is dissolved in a strong solvent (1), and then the precipitation of the dissolved polymer on the column head occurs (2) in combination with the adsorption to the stationary phase (3). By attaining a suitable solvent combination between solvent and non-solvent, e.g. the cloud-point of polymer, the precipitated polymer is re-dissolved (4) but remains adsorbed to the stationary phase (5). In the final step, the complete elution of the fully dissolved polymer occurs from the stationary phase (6). German et al [13,15,16] showed in a series of papers the differences between precipitation- / redissolution and adsorption mechanisms by analyzing polyesters. For crystalline polyester, a clear dependence on precipitation- / re-dissolution mechanism could be shown, as for all other studies adsorption effects dominated or at least supported the separation.

An overview of different possible setups for gradient elution is shown by Deyl [17] and Jandera [18]. For a first approach, the slope variation of a linear gradient is a good choice and may sometimes lead to multilinear gradients enhancing the separation. Therefore, Nikitas et al [19–23] presented various approaches for optimizing multilinear gradients. Moreover, software packages such as DryLab or PREGA use similar theoretical concepts [24]. As a consequence, concave and convex gradient shapes might also be a useful alternative [25]. Furthermore, especially for various types of macromolecules, a step gradient improves peak resolution and separation performance. The analysis of azeotropic and low-conversion poly(styrene-stat-2-methoxyethyl methacrylate) [26], styrene acrylonitrile copolymers [27], lignin [28] or humic like substances [29,30] was improved through different types of step gradients. Applying a step gradient, various isocratic steps result in an improved peak resolution, which cannot usually be achieved by a linear gradient. A further improvement of this gradient profile was presented by Kajdan et al [31] for ion chromatographic separation of recombinant proteins and by Spranger et al [32] for the separation of humic like substances with RP-HPLC. They used a kind of spiked gradient profile, where in an additional step the elution promoting solvent was reduced at the end of the original gradient step. This modification results in a much better separation performance than in previously described gradient profiles. Beside this, Morris et al [33] optimized with a saw tooth like gradient the separation of complex protein mixtures.

As the application of step gradients in polymer separation showed pretty good results [27,34], further investigations concerning the shape of the gradient profile are promising for improving the separation. In this report, a novel gradient protocol for the separation of polymers, which allows each individual solvent composition to perform its unique re-dissolution ability, is evolved. With a saw tooth gradient protocol, the separation performance significantly increased. Therefore, the aim of this work was the development of a preferably universal saw tooth gradient protocol which allows high-resolution polymer HPLC (HRP-HPLC) of different types of polymers over a broad molecular weight range.

2. Material and methods

2.1. Software

The programming of the saw tooth gradient was possible over the entire gradient range from 0 to 100% desorption promoting solvent with the chromatographic data system Chromeleon (Thermo Fisher Scientific, version 7.2), as other investigated chromatographic software packages are limited in the number of possible entries of the gradient table. Moreover, currently, a complete saw tooth gradient ranging from 0 to 100% with the corresponding steps can be achieved in combination with Thermo Fisher Scientific (Waltham, USA) HPLC pumps. The Agilent driver module of Chromeleon limits the gradient entries to 69 and Agilent ChemStation Version C limits the gradient entries to 100 for an Agilent (Waldbronn, Germany) HPLC system, while the limitation of WinGPC UniChrom 8.2 (Polymer Standard Service) is 161 entries. These limitations made it difficult to use the full potential of the saw tooth profile and, therefore, using an Agilent fraction collector only a small region of interest could be fractionated.

2.2. Hardware

2.2.1. LC systems and detectors

The optimization of the saw tooth gradient with design of experiments (DoE) by Taguchi was done on a Thermo Fisher Scientific (Waltham, USA) Vanquish UHPLC with UV detection at 215 nm and a 385 ELSD, equipped with an enhanced parallel-path MiraMist® poly(tetrafluoroethylene) nebulizer from Burgener Research Inc. (Mississauga, Ontario, Canada) at 40 °C evaporator temperature, 90 °C nebulizer temperature, and a gas flow of 1.2 SLM (standard liter per minute, see [35]). The investigated parameters for the

Table 1
Investigated parameters for the design of experiment according to Taguchi's L16(4^5) approach.

	Group	1 Poroshell C18 50 x 4.6 mm, 2.7 μm	2 Poroshell C18 100 x 4.6 mm, 2.7 μm	3 Hypersil BDS C18 100 x 4.6 mm, 2.4 μm	4 Luna C18 100 x 4.6 mm, 5 μm	5 Hypersil Gold C18 aQ 100 x 10 mm, 5 μm
Label	**Parameter**	**Level 1**	**Level 2**		**Level 3**	**Level 4**
A	height of the negative backward gradient step [%]	3.0	6.0		9.0	12.0
B	effective step height [%]	0.2	0.5		0.8	1.0
C	retardation of negative slope	0.5	1.0		2.0	3.0
D	lower plateau	0.5	1.0		2.0	3.0
E	retardation of positive slope	0.1	0.5		1.0	2.0

optimization of the saw tooth gradient profile by DoE are given in Table 1 and were performed from 0% THF (100% methanol) to 100% THF (0% methanol) considering different types of stationary phases.

The other analytical measurements of different types of polymers were performed on an Ultimate 3000 HPLC of Thermo Fisher Scientic with the modified 385 ELSD.

2.2.2. Studies of the real shape of the gradient profile

The actual gradient profile was measured with an Ultimate 3000 diode array detector at 265 nm, following the recommendation of Thermo Fisher Scientific for operational/ performance qualification (OQ/PQ) for gradient accuracy with 100% pure water as starting condition against 0.1% acetone in water [36]. As test columns an Agilent Poroshell C18 EC (50 x 4.6 mm, 2.7 μm), an Agilent Poroshell C18 SB (150 x 4.6 mm, 2.7 μm), and a restriction capillary of 15 m length and 0.18 mm ID from OQ/PQ Kit of Thermo Fisher Scientific were used.

2.2.3. Semi preparative LC systems

Fraction collection of PVC was performed on an Agilent 1100 series LC system with a THF resistant 3115α degasser from ERC (Riemerling, Germany) equipped with an Agilent fraction collector. For adjusting the separation pattern at an Agilent Poroshell C18 EC (50 x 4.6 mm), a 385 ELSD modified with an enhanced parallel-path MiraMist® poly(tetrafluoroethylene) nebulizer from Burgener Research Inc. (Mississauga, Ontario, Canada) was used at 40 °C evaporator temperature, 90 °C nebulizer temperature, and 1.2 SLM gas flow. The LC flow rate was set to 1.0 mL min^{-1} and the injection volume was 10 μL (1 mg absolute sample amount). The saw tooth gradient was started at 26% THF and 74% methanol and ended at 56% THF and 44% methanol with an effective step height of 2% and a height of the negative backward gradient step of 9%. After each separation, the column was flushed with 100% THF for 8 min in order to remove not eluted polymer from the column. For sufficient amount of sample per fraction, 50 injections were performed. The SEC measurements were performed with an Agilent 1260 SEC system and a Shodex (Munich, Germany) RI101 detector on a set of four Styragel® columns (HR1, HR3, HR4 and HR5, Waters, Eschborn, Germany) and THF as eluent at 1.2 mL min^{-1}.

Fraction collection of PS was performed on a 1260 series LC system of Agilent with a THF resistant 3115α degasser from ERC (Riemerling, Germany). UV detection was performed at a wavelength of 215 nm A Poroshell C18 EC (50 x 4.6 mm) was used as stationary phase. The gradient profile was started at 0% THF and 100% methanol, within 15 min a linear gradient was set to 31% THF and then the actual saw tooth gradient started with an effective step height of 0.2% and 9% height of the negative backward gradient step up to a final concentration of 39% THF and 61% methanol. After each separation, the column was flushed with 100% THF for 8 min in order to remove not eluted polymer from the column. A flow rate of 1.0 mL min^{-1} and an injection volume of 15 μL (0.75 mg absolute sample amount) were applied. To get sufficient sample amount per fraction 100 runs were performed. For evaluation of the collected fractions MALDI-ToF-MS measurements were performed on a Shimadzu Axima Performance MALDI-ToF-MS (Kratos, Manchester, UK). As cationization reagent a solution of 100 mol L^{-1} sodium trifluoroacetate (Sigma-Aldrich, Darmstadt, Germany) in THF was used and 10 mg mL^{-1} trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) (Sigma-Aldrich) was used as MALDI matrix. Various mixing ratios between sample solution: matrix solution: cationization solution of 10:10:1, 10:20:10, 10:50:1 and 10:100:1 (v/v/v) were used in order to obtain an appropriate spectrum because the actual concentrations of the collected fractions were unknown.

Fraction collection of PDMS with a viscosity of 350 mPa·s was performed on a 1260 series LC system from Agilent with a THF resistant 3115α degasser from ERC. For adjusting the separation pattern, a 385 ELSD modified with an enhanced parallel-path MiraMist® poly(tetrafluoroethylene) nebulizer from Burgener Research Inc. was used at 40 °C evaporator temperature, 90 °C nebulizer temperature, and 1.2 SLM gas flow. An Accucore C18 (50 x 4.6 mm) column was used, the LC flow rate was set to 1.0 mL min^{-1} and 0.4 mg sample amount were injected 100 times, to get sufficient amount of sample per fraction, for separation with a saw tooth gradient (effective step height 1.0%, effective step length 1.50 min) and a linear gradient with methanol and THF as mobile phase components, respectively. The multilinear gradient was started at 100% methanol and reached 30% THF after 15 min, 65% THF after 78 min, and 100% THF after 79 min. Each fraction (16 fractions, starting from 37 min up to 61 min) was collected within 1.50 min intervalls, for both gradients. For fractionation evaluation, MALDI-ToF-MS measurements were performed according to the above-mentioned protocol, but in place of sodium trifluoroacetate, silver trifluoroacetate was used as cationization reagent.

2.3. Stationary phases

For preparative fraction collection of PS and PVC, an Agilent Poroshell C18 EC (50 x 4.6 mm, 2.7 μm) was used. For the DoE approach (see Table 2) an Agilent Poroshell C18 EC (50 x 4.6 mm, 2.7 μm), an Agilent Poroshell C18 EC (100 x 4.6 mm, 2.7 μm), a Thermo Fisher Scientific Hypersil BDS C18 (100 x 4.6 mm, 2.4 μm), a Thermo Fisher Scientific Hypersil Gold C18 aQ (100 x 10 mm, 5 μm), and a Phenomenex (Torrance, USA) Luna C18 (100 x 4.6 mm, 5 μm) were used as superimposed group for the parameters of the saw tooth gradient profile.

The investigations of various polymer standards were done under optimized gradient conditions on a Thermo Fisher Scientific Accucore C18 (50 x 4.6 mm, 2.6 μm) and an Agilent Poroshell HILIC (50 x 4.6 mm, 2.7 μm).

Table 2
DoE confirmation experiments for different stationary phases for the optimum shape settings of the saw tooth gradient.

Column	A [%]	B [%]	C	D	E
Poroshell C18 50 x 4.6 mm, 2.7 µm	6.0	0.2	1.0	3.0	2.0
Poroshell C18 100 x 4.6 mm, 2.7 µm	6.0	0.2	0.5	2.0	2.0
Hypersil BDS C18 100 x 4.6 mm, 2.4 µm	6.0	0.2	1.0	3.0	2.0
Luna C18 100 x 4.6 mm, 5 µm	6.0	0.2	1.0	3.0	2.0
Hypersil Gold C18 aQ 100 x 10 mm, 5 µm	6.0	0.2	0.5	0.5	2.0
ideal settings independently of column type	6.0	0.2	1.0	3.0	2.0

Table 3
Overview of used polymer standards.

Polymer	M_p [g·mol^{-1}]	Polydispersity
PS 8995	8995	1.03
PS 19600	19600	1.02
PVC 23900	23900	1.21
PVC 45400	45400	1.30
PVC 92100	92100	1.32
PVC 202000	202000	1.34
PDMS 1300	1300	1.34
PDMS 2000	2000	1.42
PDMS 5400	5400	1.67
PDMS 8300	8300	1.83
PDMS 20700	20700	3.02
PDMS 36500	36500	2.98
PDMS 71200	71200	4.35
PDMS 130000	130000	6.09
PDMS 250000	250000	10.94
PMMA 19700	19700	1.09
PMMA 107000	107000	1.1
PMMA 690000	690000	1.09
PMMA 1600000	1600000	1.33
PPG 4850	4850	1.10
PPG 13300	13300	1.14
PPG 19600	19600	1.25
PPG 27100	27100	1.61

2.4. Polymer samples and chemicals

All solvents used were HPLC grade. Acetone, acetonitrile (ACN), methanol (MeOH), toluene, n-hexane, and non-stabilized tetrahydrofuran (THF) were purchased from Merck (Darmstadt, Germany) and used without further purification. Water of a Milli-Q-Advantage A10 water system (Merck Millipore) was used. The analyzed polymer standards and samples are summarized in Table 3. PS 19 600 standard was purchased from PSS (Mainz, Germany), PDMS standards were obtained from Wacker Chemie AG (Burghausen, Germany), PPG standards were purchased from American Polymer Standards Corporation (Mentor, OH, USA) and all other standards listed in Table 3 were purchased from Agilent (Church Stretton, UK).

3. Results and discussion

Beginning with linear and stepwise gradients, the development of a saw tooth like gradient profile was done for improving separation performance of polymer HPLC. For optimization of the new gradient profile, design of experiments (DoE) according to Taguchi's approach were applied. Additionally, the limitations of the concept regarding laminar flow profiles and therefore mixing accuracy were evaluated. The separation performance of the saw tooth gradient was studied by heart-cut fraction collection with subsequent MALDI-ToF-MS or SEC measurements of each fraction. Finally, the universal application of this high-resolution polymer HPLC (HRP-HPLC) approach to various types of polymers, e.g. PS, PVC, PMMA, PDMS, and PPG, was demonstrated.

3.1. Fundamental studies of the saw tooth gradient

3.1.1. Development – from linear gradient to saw tooth gradient

As aforementioned, the resolution of HPLC for polymer analysis especially in the high molecular mass region is limited. In adsorption dominated gradient separation, no separation according to molecular mass is achievable above the point of critical adsorption. Only by means of a dominating precipitation- / re-dissolution mechanism or by absence of a point of critical adsorption further separation in this higher molecular mass range are possible [1]. Our current research is primarily directed to the yet unresolved, or rather poorly resolved molecular mass range above low molecular oligomer separation. In case of the investigated PVC standards (molar masses in peak maximum from 23,900 g mol^{-1} up to 202,000 g·mol^{-1}, see Table 3) with linear gradients in polymer HPLC, a poorly resolved peak could be measured. The multimodalities in the peak were also present on other stationary phases, other solvent combinations or at injecting lower sample amounts. Therefore, even by optimizing mobile and stationary phases, no significant improvements were possible. Comparing a high-resolution stepwise gradient with 0.2% step height with a linear gradient starting from 100% MeOH to finally 100% THF did not show any appreciable differences (see Fig. 1 a + b for PVC 45,400). Applying the saw tooth gradient, where in an additionally step a negative gradient slope interrupts the elution of the polymer, resolution was significantly improved (see Fig. 1 c). The back and forth strategy of the gradient profile led to repeated fractionated elution steps, enabling selective elution of different polymer fractions. Therefore, the improvement of resolution, which was highly reproducible (compare, e.g. the set of measurements described in chapter 3.2.1 for preparative studies), was achieved to get a more detailed overview of the investigated polymer samples. An actual oligomer separation was not the primary target. According to these experimental results, two main questions arose:

1) Was this a real separation result or the recording of artefacts corresponding to the chosen gradient profile?
2) What is the optimum adjustment of the saw tooth profile and where are the limitations?

A detailed answer to the first question will be given in chapter 3.2.1 and briefly, it is a HPLC-like separation from low to high molecular masses. For the second question, further considerations were necessary which were regarded in a DoE by gradient specific parameters. Furthermore, the accuracy of mixing system and gradient profiles were examined.

3.1.2. Investigation of the gradient profile of the saw tooth approach

3.1.2.1. Design of experiments according to the methodology of Taguchi.

The optimization measurements were carried out with a PS standard of molar mass at peak maximum of 19 600 g mol^{-1}. Methanol was used as weaker or rather non-solvent and THF as strong or desorption promoting solvent with a gradient range from 0.0 to 100.0% THF (100.0 to 0.0% MeOH). Five different types of C18 columns

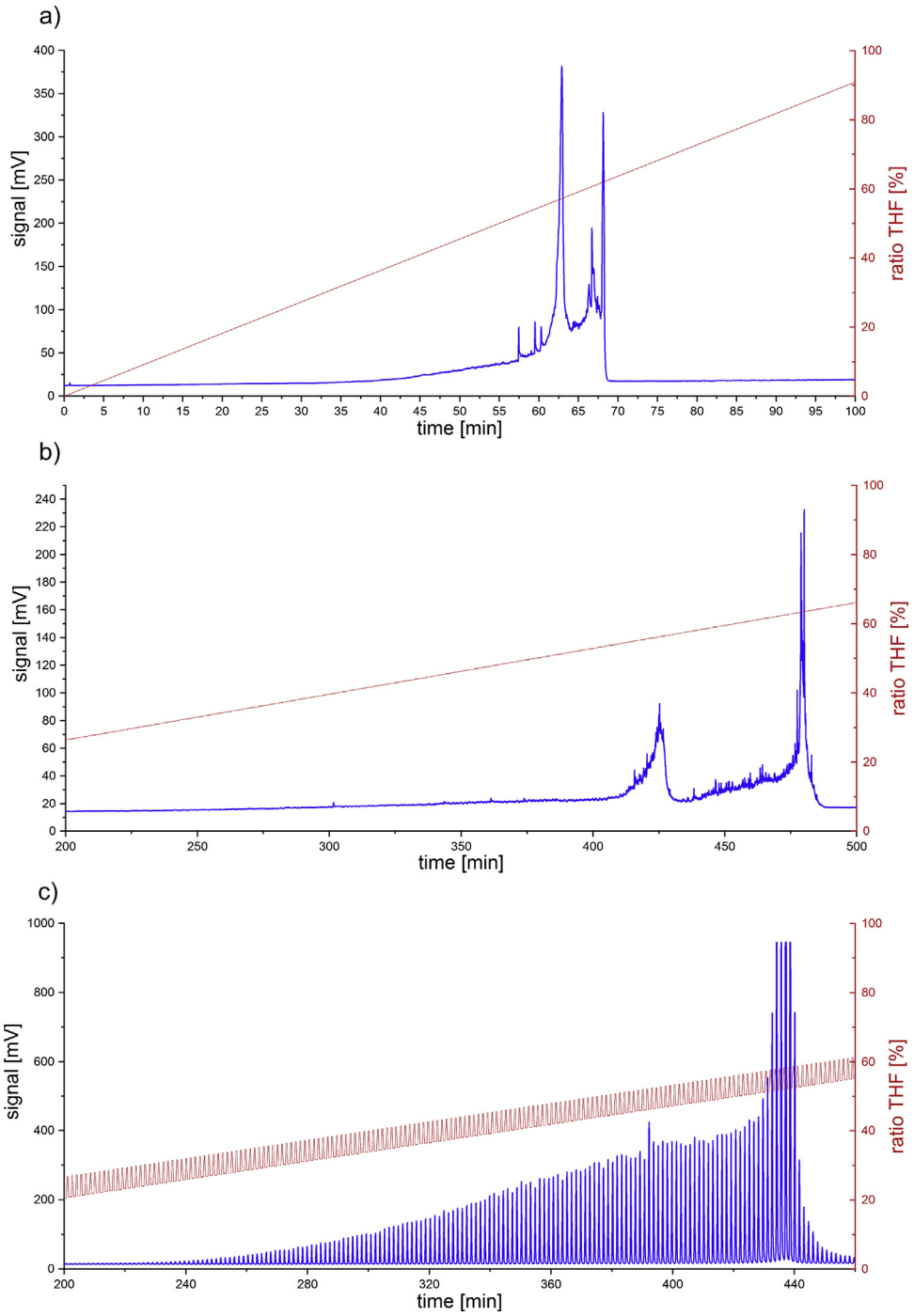

Fig. 1. Development of saw tooth gradient profile for the separation of PVC 45400 on an Accucore C18 (50 x 4.6 mm, 2.6 μm) column with MeOH as weaker or rather non-solvent and THF as desorption promoting solvent; detection with ELSD; chromatograms corresponding to a linear gradient (a), a stepwise gradient with step length (b) of 1.5 min and step height of 0.2% and a saw tooth gradient with effective step length of 1.5 min and effective step height of 0.2% (c).

were used varying in length, internal diameter, particle size and total porous particles as well as superficially porous particles (see Table 1).

Using DoE, instead of one-factor-at-a-time offers the advantage of simultaneously varying several parameters and, thus, reducing the number of necessary experiments [37,38]. According to Taguchi's transformation of the response value into a signal-to-noise value, the variability of the different types of columns was included in the evaluation resulting in a higher reliability and optimization. Particularly, the advantage of Taguchi's approach is the reduced number of experiments necessary for considering the investigated parameters at different levels when compared to other approaches used in chemometrics. Furthermore, in Taguchi's approach the influence of a disturbance on the system is minimized without eliminating its reason. Further information of Taguchi's methodology is given in [39–41]. Fig. 2 gives an overview of the investigated and optimized parameters. The parameters *A* and *B* describe the ratio of desorption promoting solvent THF in the different steps of the saw tooth profile: *A* depicts the height of the negative backward gradient step, and *B* the effective step height between the consecutive upper plateaus. Initially, the experiments started based on interstitial column volumes using the column vol-

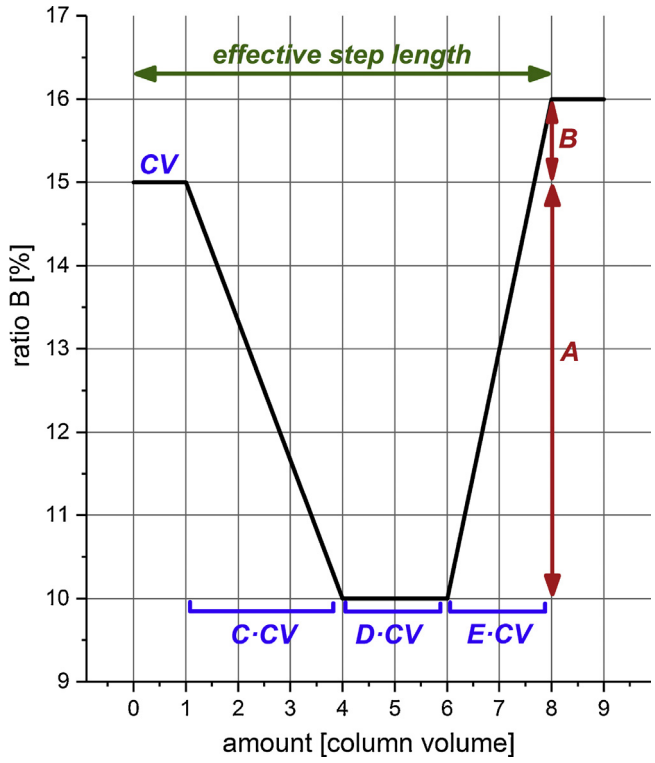

Fig. 2. Scheme of a general saw tooth gradient protocol, presented at one explicit effective step length and described by the amounts of column volumes (CV). Parameter *A* [in %] represents the height of the negative backward gradient step for the drop of the mobile phase composition, *B* [in %] represents the effective step height of the saw tooth gradient; the variation in several regions of the saw tooth shape is described by parameters *C*, *D* and *E*, as retardation of negative slope, duration of the lower plateau, and retardation of the positive slope, respectively.

ume (CV) as scale up / down factor. The parameters *C*, *D*, and *E* determine the step length of the retardation of the negative slope, the one of the lower plateau and the one of the retardation of the positive slope, respectively. The investigated parameters and corresponding levels of Taguchi's L16 (4^5) are summarized in Table 1. Data evaluation based on number of peaks detected by the experiment, lowest peak resolution, asymmetry and peak width at half height of the highest peak. The confirmation experiments considering the five investigated stationary C18 phases are presented in Table 2. The confirmation experiments considered each column alone as well as all columns together resulting in a set of parameters, which are independent of column dimension, particle size, or type. These observations were in good agreement with the literature [1,13]. The prominent response factors for the saw tooth gradient profile are number of discriminable peaks, the larger the better, peak resolution, also larger the better because the inherent limited resolution of polymer peaks, and peak asymmetry, which should be around one. As depicted in Table 2 the height of the negative backward gradient step (parameter *A*), effective step height (parameter *B*) and retardation of positive slope (parameter *E*) show the same behavior at all investigated columns. Parameter *B*, the effective step height alone, dominates the number of peaks and the peak height while parameter *A* accounts for asymmetry and resolution. Furthermore, the retardation of the positive slope (parameter *E*) was ideal at its highest investigated level of one column volume while the retardation of the negative slope (parameter C) should be one column volume. The length of the lower plateau (parameter *D*) shows the greatest variability, especially in considering analytical and semi-preparative columns. However, due to the analyses of variances (ANOVA), this parameter is only of minor significance for the saw tooth gradient profile and, thus, can be arbitrarily chosen. Although not considered by the DoE, the time of measurement should always be kept as short as possible maintaining a reasonable peak resolution. The number of steps applied in the saw tooth gradient directly affects the run time: If high resolution with maximum number of peaks is sought, the corresponding run time will be rather long. For the investigated types of columns, a set of parameters can be chosen being independent of the type of particles and column dimensions allowing a nearly universal approach. Based on these results, it is assumed that the back- and forth change in solvent composition, which caused the fractionated elution, is in general the most effective parameter for the enhancement of the separation.

3.1.2.2. Constraints by sample loading per injection.

The saw tooth gradient basically depends on consistency of the programmed gradient profile to the actual gradient because of mixing accuracy and the system diffusion. The actual gradient profile was evaluated by measurements based on 0.1% acetone in water against pure water at 265 nm detection wavelength. In a first approach, the effect of various column dimensions compared to a restriction capillary of 15 m x 180 μm was studied concerning the actual gradient profile. On basis of a very symmetric (which means each gradient step is of equal duration) saw tooth gradient a pretty good correlation or rather similar shape between calculated and effective profile was found, independently of the column dimensions or pathway within the LC system (Fig. 3a). The major drawback of such a saw tooth gradient was its very poor separation performance. If the parameter settings obtained in a DoE are optimal, a more asymmetric gradient profile is necessary for a better separation performance. Applying a generic optimal gradient setting (Fig. 3 b + c) by only varying the effective step length (cf. Fig. 1) the overlay between calculated and actual gradient showed distinct deviations. Fig. 3b and c depict the impact of effective step height on the match or mismatch between both curves. No considerable dependence of the effective step height was noticeable between both measurement series (Fig. 3b vs. c). At effective step lengths above 0.60 min (chromatograms IV – VI) the alignment between actual and calculated gradient curve at the right edge (at increasing positive slope) improved as well as the separation performance. Besides an appropriate congruence of theoretical and practical gradient profile, the analysis time should possibly be shortened. Thus, the gradient setup IV in Fig. 3 represented a good compromise between profile alignment and analysis time. For the specification of the optimized saw tooth gradient settings and for illustrating the calculation of different possible saw tooth gradients, a Microsoft Excel file is shown in the supplementary material (S1).

A further important influencing factor on peak resolution is the amount of polymer sample. In Fig. 4 the impact of injection volume and, thus the sample amount of PMDS is shown. Similar results were found while varying sample concentration at constant injection volume. The height of the small double-headed arrow in Fig. 4 represents a qualifier of separation performance. For injection volumes up to 5 μL or absolute sample amounts up to 100 μg nearly baseline resolved polymer peaks were obtained on the investigated stationary phase, while for higher sample amounts a prolonged effective step length would be necessary.

As conclusion, sample concentration or respectively absolute sample amount had to be adjusted carefully to the saw tooth gradient profile to achieve a good separation performance in the shortest possible analysis time. In addition to general overloading effects, caused by oversized injection volumes or absolute sample amounts with respect to stationary or mobile phase, it is important to avoid mass overloading and volume overloading with respect to the gradient profile.

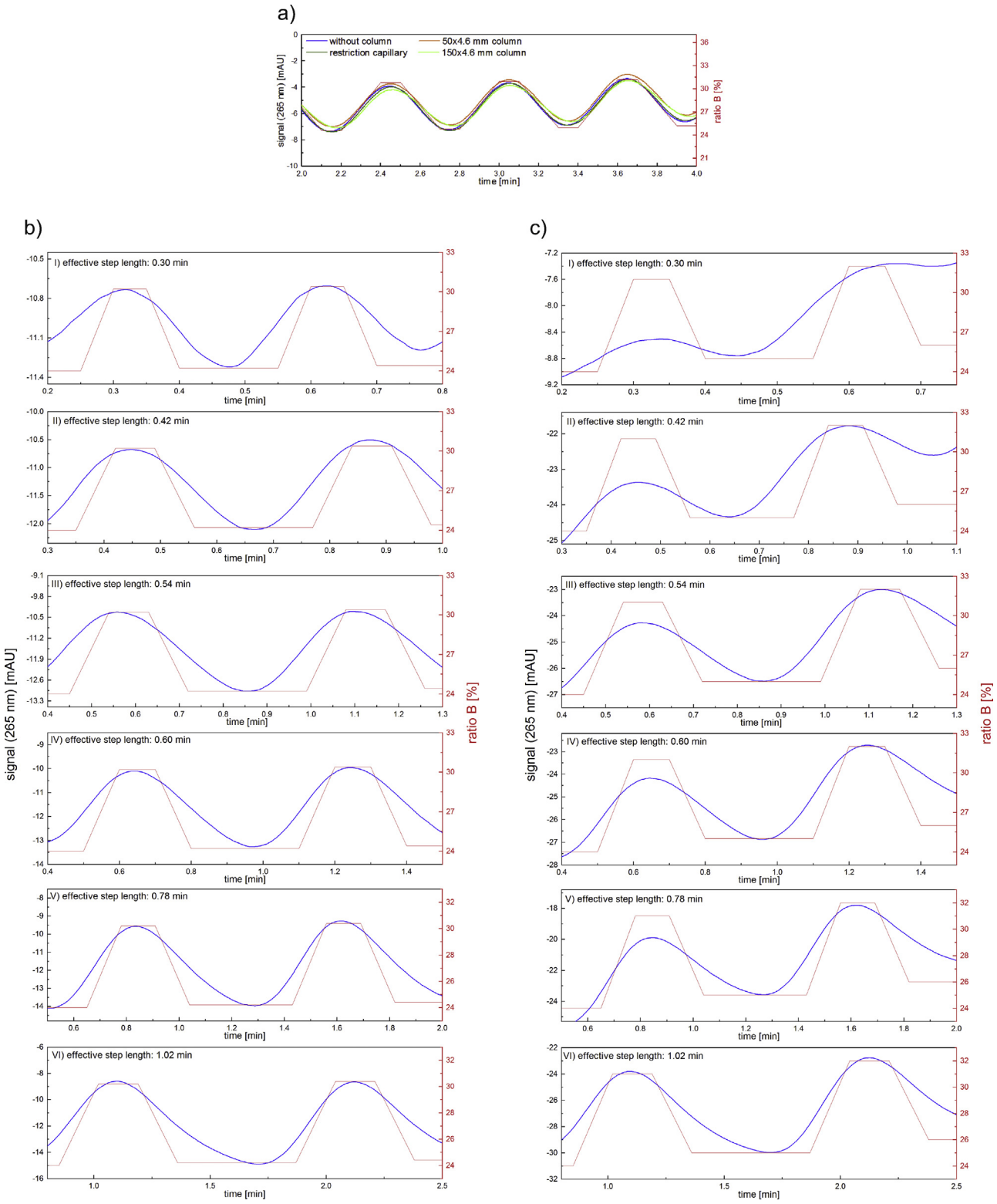

Fig. 3. Comparison between programmed saw tooth profile and real gradient shape at different columns and step lengths of the saw tooth profile; a) shows the overlay of the programmed almost symmetric (each step of similar length) saw tooth gradient with a restriction capillary, a 50 x 4.6 mm column, a 150 x 4.6 mm column and without column, the distinctions depended on the effective step length between the programmed and the actual gradient profile are shown for 0.2% (b) and 1.0% (c) effective step height; measurements were done according to the PQ/OQ of Thermo Fisher Scientific [36] for gradient accuracy.

3.2. Application to polymer samples

With the developed and optimized saw tooth gradient, several polymer standards, e.g. PS, PVC, PMMA, PDMS, and PPG were studied. PS, PVC, and PDMS were further investigated by fraction collection evaluating the degree of separation of the applied saw tooth gradient profile while for the other polymers just the applicability was demonstrated. Methanol and THF were used as eluents;

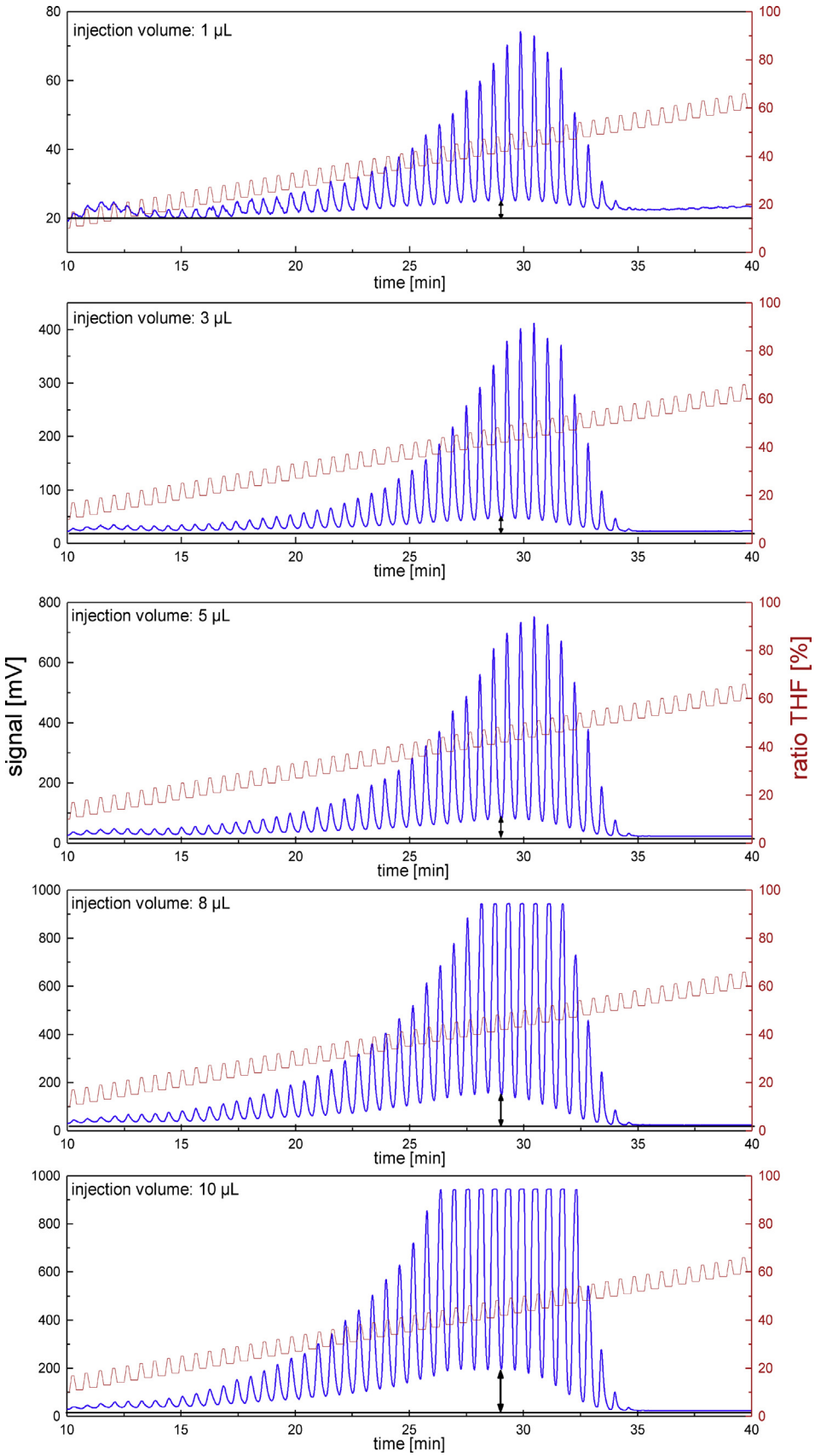

Fig. 4. Variation of injected sample amount and influence on the separation performance of the saw tooth gradient, showed for PDMS of viscosity of 1000 mPa·s and a sample concentration of 20 mg mL^{-1}; Measurements were done with MeOH as weaker or non-solvent and THF as desorption promoting solvent on a Poroshell HILIC (50 x 4.6 mm, 2.6 µm).

acetonitrile as weak or non-solvent did not show any significant advantage compared to methanol. Substituting THF as desorption promoting solvent was not investigated because of extraordinary dissolving properties of THF for the used polymers.

3.2.1. Preparative HRP-HPLC

For fraction collection by heart-cut technique, polymeric standards were chosen with a polydispersity of about 1.1. to 1.5. Re-analyzing each single fraction showed that separation depended on molecular mass differences: The low molecular mass analytes elute first and with elution time the molar masses increase.

3.2.1.1. Heart-cut HRP-HPLC x SEC for PVC analysis.

As depicted in Fig. 5 PVC was fractionated on a Poroshell C18 EC with methanol and THF as eluents. The effective step height of the saw tooth gradient was 2.0%, simplifying the gradient profile and reducing the necessary time as well as overcoming the restraints due to limited entries in the gradient timetable for the used LC system. Afterwards, the 15 collected fractions were investigated by SEC and the results are presented in Table 4. Compared to the original PVC sample of a specified molar mass of M_p = 23,900 g mol^{-1}, the polydispersity of all fractions became narrower from fraction 1 up to fraction 15. These results proved that the HRP-HPLC separation of the investigated homopolymer only depended on the molecular weight. As consequence, the separation using the saw tooth gradient profile is a real separation and not caused by artefacts (to answer question 1 from Section 3.1.1). This novel approach to the analysis of synthetic polymers considerably improved the separation performance for polymeric samples.

3.2.1.2. Heart-cut HRP-HPLC x MALDI-ToF-MS for polystyrene analysis.

For the separation of polystyrene, an extended saw tooth gradient with an effective step height of 0.2% was chosen. Throughout 20 injections of PS with molar mass of Mp = 8995 g·mol^{-1}, (50 mg mL^{-1}, 15 μL) a continuous increase of column backpressure occurred. Flushing the column with THF, toluene, or n-hexane as well as flushing the column in reversed direction overnight did not reduce the high backpressure. Thus, it was assumed that the analytical stationary phase was not an ideal choice for semi-preparative HPLC measurements with such high sample loads on the column. The same characteristics were observed for the later (chapter 3.2.2) mentioned PDMS sample with an analytical Accucore C18 column. Therefore, further research will be done with different types of stationary phases, which are more suitable for preparative separation. Fig. 6 depicts the chromatogram obtained with the corresponding saw tooth gradient and the collected fractions, which were measured by MALDI-ToF-MS (Table 5 and S2a-d). Compared to the SEC measurements for PVC analysis, where polydispersity might be overestimated through band broadening effects, MALDI-ToF-MS often leads to under estimating polydispersity due to discrimination by the ionization process [42]. In this study, beside these differences, both techniques led to the same conclusion of decreasing polydispersities by fractionation with a saw tooth gradient. Beginning from fraction one to twenty the molar mass continuously increased, while this was not found for higher fraction numbers. Presumably, the separation performance was reduced because of the use of the analytical stationary phase, which was not ideal for semi-preparative separation with the applied system. Especially the used high sample amounts may have caused these problems. Nevertheless, by heart-cut fraction collection it was proven that PVC and PS were separated due to their differences in molar mass caused by the same principle of separation.

3.2.2. Application to various polymer types

All measurements of Fig. 7 and Supplementary Material S3 were performed with a reduced resolution associated with choosing 1% effective step height in the saw tooth gradient reducing the runtime. An economical approach is first to apply a saw tooth gradient with low resolution for obtaining an overview and then applying a gradient with higher resolution if necessary. For several polymers, e.g. PVC (Fig. 7a), PMMA (Fig. 7b), PDMS (Fig. 7c) and PPG (Fig. 7d), screening measurements were performed. In each case, the determination was done on an Accucore C18 column with methanol as weaker or non-solvent and THF as desorption promoting solvent. In future measurement series, the influence of different weaker or non-solvents with respect to the dissolving property of the investigated polymers will be further investigated. For comparison, the inset on the left-hand side of Fig. 7 shows the separation performance of a linear gradient, respectively. Evaluating the performance of preparative linear and saw tooth gradients on analytical columns, PDMS with an average molar mass of 20,800 g mol^{-1} was fractionated with a screening saw tooth gradient of 1.0% effective step height and a corresponding linear gradient. Subsequent analysis of the fractions at the maximum of the original PDMS distribution (about M_P = 22,000 g·mol^{-1}) with MALDI-ToF-MS showed even for the screening saw tooth gradient an improved separation performance (fraction 13, Supplementary material, S4). Apart from a more symmetric polymer distribution, the saw tooth gradient resulted in a better separation, particularly at the boundaries (compare the inlets in S4) of the polymer distribution. For the fraction of the linear gradient, the mass resolution was decreased over the whole mass spectrum. Furthermore, in the mass range between 24,000 and 25,000 g·mol^{-1} a shoulder in the distribution occurred. This might be caused by a more unprecise fractionation at the linear gradient compared to the saw tooth gradient. Based on these results, further research with semi-preparative equipment is planned to show further applicability of the combination of saw tooth gradient and heart-cut two-dimensional liquid chromatography.

The main contribution of the stationary phase is primarily its capability of retention of the analyte. Replacing a C18 column by a Poroshell HILIC column (c.f. supplementary material S3) PVC, PMMA and PDMS showed the same separation behavior. Interestingly, PPG could not be separated on a HILIC column applying the same conditions because of missing retention. However, comparing C18 with HILIC for HRP-HPLC, peak resolution of various polymers corresponded to each other and showed the minor significance of stationary phase in HRP-HPLC. Adapting an appropriate separation system, e.g. eluent combination, nearly each homopolymer can be separated in a distinct peak distribution. In fractionating polymers of the same kind, the distribution can be simplified for further investigations with other techniques such as mass spectrometry or size exclusion chromatography. Particularly, for the molar mass range greater than 200,000 g mol^{-1} an unprecedented separation performance regarding peak resolution was achieved by HRP-HPLC (cf. Fig. 8).

4. Conclusion

Based on GPEC and HPPLC a novel technique termed high-resolution polymer HPLC (HRP-HPLC) was introduced. The HRP-HPLC is based on the application of a saw tooth gradient profile, which was developed, optimized, and validated for analysis of polymers. The profile of an optimum saw tooth gradient was evaluated by design of experiments. Special attention has to be taken choosing the appropriate sample amount for injection because peak resolution and effective step length of the gradient profile depend on the sample concentration. Regarding analysis

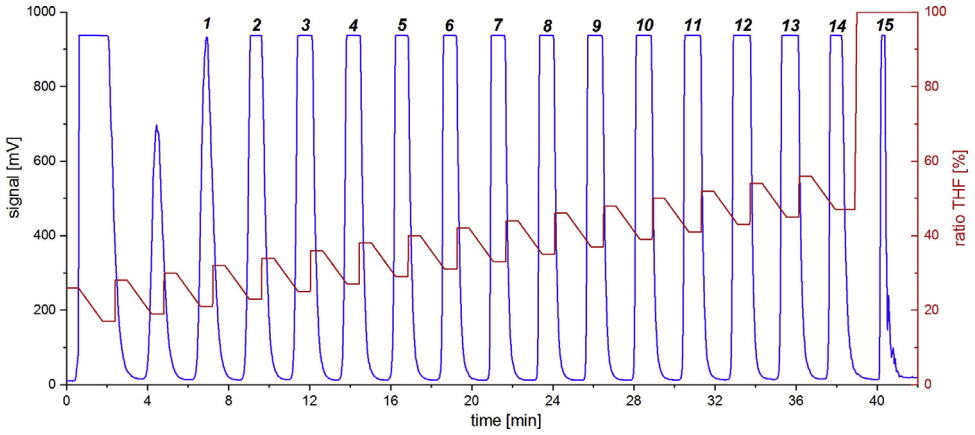

Fig. 5. Chromatogram and cutting pattern for preparative PVC analysis, PVC 23900 (c = 115 mg·mL^{-1}) was analyzed with a saw tooth gradient of 2.0% effective step height, because of the limitation to only 69 gradient time table entries for the used HPLC pump; analysis was done with MeOH as weaker or non-solvent and THF as desorption promoting solvent on a Poroshell 50 x 4.6 mm Poroshell C18 EC; fractions 1–15 were analyzed with SEC.

Table 4
Results of SEC measurements after separation and fraction collection of PVC 23900 with the saw tooth gradient (determined on a set of Waters Styragel® (HR1, HR3, HR4 and HR5) with THF as eluent at 1.2 mL min^{-1}).

Sample name	M_w [g·mol^{-1}]	M_n [g·mol^{-1}]	Polydispersity	M_z [g·mol^{-1}]	M_p [g·mol^{-1}]
PVC 23900	26 000	20 600	1.26	30 500	28 100
F1	18 600	17 100	1.08	19 900	18 400
F2	20 300	18 500	1.10	21 900	20 700
F3	22 900	20 600	1.11	24 800	23 800
F4	24 900	22 200	1.12	27 400	26 100
F5	26 400	23 200	1.14	29 300	27 000
F6	27 000	23 600	1.14	30 100	27 600
F7	27 700	24 200	1.15	30 900	28 200
F8	28 400	24 700	1.15	31 700	29 000
F9	28 800	25 100	1.15	32 300	29 300
F10	29 400	25 600	1.15	33 000	30 200
F11	29 900	25 800	1.16	33 700	30 700
F12	29 900	25 700	1.16	33 800	30 700
F13	30 500	26 300	1.16	34 400	31 300
F14	30 600	26 400	1.16	34 500	31 300
F15	31 600	27 200	1.16	36 200	32 500

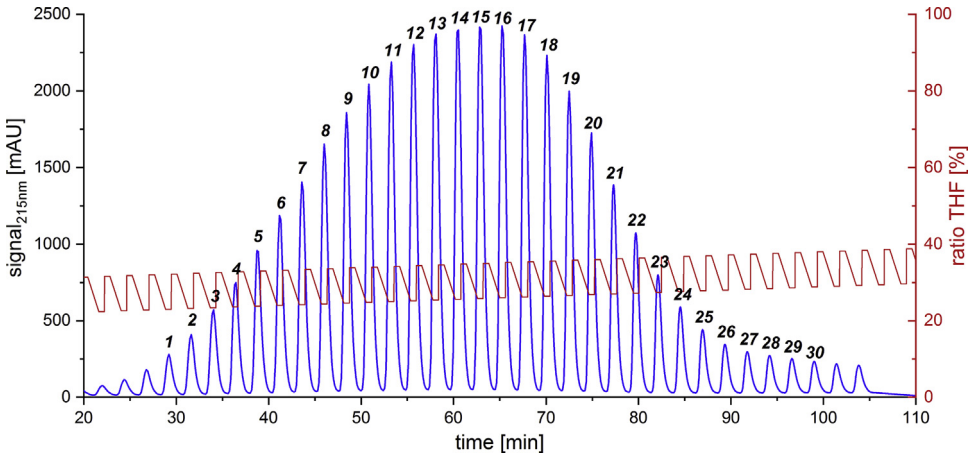

Fig. 6. Chromatogram and cutting pattern for preparative PS analysis, PS 8995 (c = 50 mg·mL^{-1}) was analyzed with a saw tooth gradient of 0.2% effective step height, limited by the given mixing accuracy of the used HPLC pump; analysis was done with MeOH as weaker or non-solvent and THF as desorption promoting solvent on a Poroshell C18 EC (50 x 4.6 mm, 2.7 μm); fractions 1–30 were analyzed with MALDI.

time, a screening approach with reduced run time and resolution or a high-resolution approach with an extended run time can be chosen by only adjusting the effective step height of the gradient profile. Compared to common liquid chromatographic methods such as SEC, HRP-HPLC is characterized by a superior resolution especially in the high molecular mass range. Despite the highly increased resolution, the new gradient technique currently does not allow a separation of single oligomers. However, the number of oligomers per single saw tooth gradient step could be considerably reduced through the fractionated elution. A major constraint of typical chromatographic software packages is the possibility of generating gradient tables with up to 2000 entries for exploita-

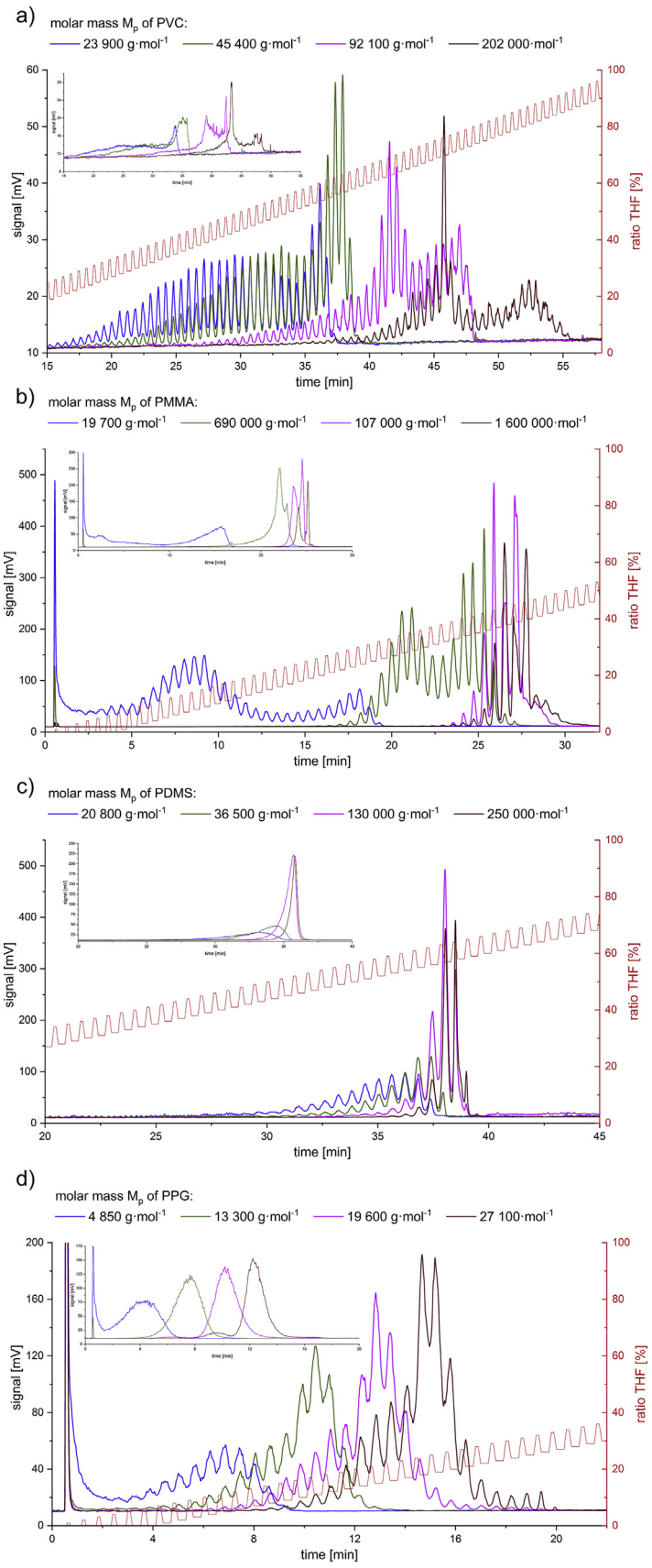

Fig. 7. Application of screening saw tooth gradients (effective step length of 0.6 min, effective step height 1.0%) for separation of various polymer types over a broad molecular weight range, the picture-in-picture chromatogram shows the separation with application of a standard linear gradient; HRP-HPLC applied to PVC (a), PMMA (b), PDMS (c) and PPG (d) on an Accucore C18 (50 x 4.6 mm, 2.6 μm) column with MeOH as weaker or non-solvent and THF as desorption promoting solvent.

Table 5
MALDI-TOF-MS-Results of fraction collection of PS8995 after HRP-HPLC.

Sample name	M_w [g·mol^{-1}]	M_n [g·mol^{-1}]	Poly-dispersity	M_z [g·mol^{-1}]	M_P [g·mol^{-1}]
FC01	7427	7385	1.0057	7469	7453
FC02	7589	7550	1.0051	7627	7662
FC03	7726	7690	1.0048	7763	7664
FC04	7873	7838	1.0045	7910	7873
FC05	8044	8009	1.0043	8079	7975
FC06	8214	8180	1.0042	8249	8183
FC07	8272	8241	1.0038	8303	8186
FC08	8404	8374	1.0035	8434	8290
FC09	8497	8467	1.0035	8528	8288
FC10	8709	8681	1.0033	8738	8497
FC11	8844	8816	1.0031	8871	8600
FC12	8860	8833	1.0031	8888	8704
FC13	9077	9051	1.0029	9103	9019
FC14	9166	9136	1.0033	9197	9125
FC15	9370	9340	1.0031	9398	9331
FC16	9499	9468	1.0032	9528	9644
FC17	9639	9604	1.0036	9671	9855
FC18	9713	9669	1.0046	9754	9956
FC19	9912	9860	1.0053	9961	10270
FC20	10009	9944	1.0065	10069	10478
FC21	10073	9994	1.0079	10146	10374
FC22	9799	9689	1.0114	9905	9643
FC23	9882	9753	1.0131	10003	9643
FC24	9629	9504	1.0131	9753	9122
FC25	9426	9311	1.0123	9541	9331
FC26	9160	9070	1.0099	9249	8917
FC27	9174	9090	1.0092	9258	9125
FC28	9307	9216	1.0099	9396	9226
FC29	9146	9064	1.0091	9227	9123
FC30	9244	9154	1.0098	9331	9120
PS 8995	8939	8855	1.0094	9020	8914

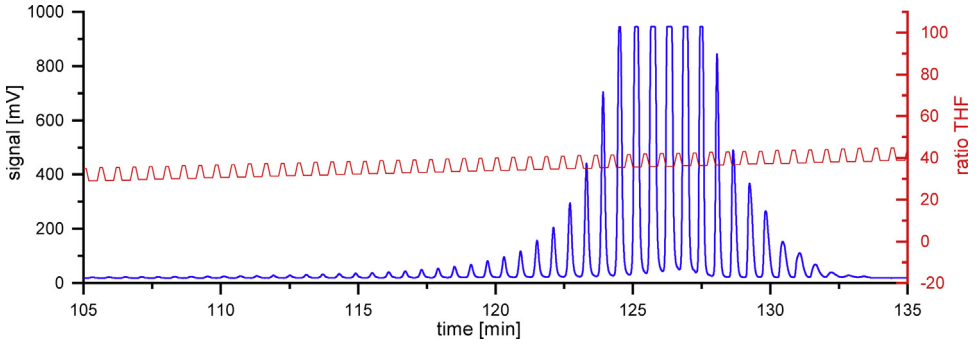

Fig. 8. High-resolution saw tooth gradient applied to PMMA 1,600,000 on an Accucore C18 (50 x 4.6 mm, 2.6 μm) with methanol as weaker or non-solvent and THF as desorption promoting solvent.

tion of the entire potential of this technique – from 100% weaker or non-solvent to 100% stronger or desorption promoting solvent with 0.2% effective step height. Presently, this approach for example is possible with Chromeleon 7.2.2 in combination with HPLC systems from Thermo Fisher Scientific. Preparative HRP-HPLC on analytical columns showed some limitations concerning sample amount, run-time, and column overloading. Therefore, further improvements of the preparative measurements are in progress. The universal applicability of HRP-HPLC was demonstrated by the separation of various types of polymers, e.g. PVC, PDMS, PMMA, or PPG, using a conventional "ordinary" HPLC system. In conclusion, the newly developed HRP-HPLC paves the way for comprehensive studies of polymeric materials.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2018.11.075.

References

[1] Y. Brun, C.J. Rasmussen, Interaction polymer chromatography, in: S. Fanali, P.R. Haddad, C.F. Poole, M.-L. Riekkola (Eds.), Liquid Chromatography: Fundamentals and Instrumentation, 2nd ed., Elsevier, Amsterdam, Netherlands, Oxford, United Kingdom, Cambridge, MA, United States, 2017, pp. 275–318.

[2] M. Schollenberger, W. Radke, SEC-Gradients, an alternative approach to polymer gradient chromatography: 1. Proof of the concept, Polymer 52 (15) (2011) 3259–3262.

[3] H. Pasch, B. Trathnigg, Multidimensional HPLC of Polymers, Springer, Berlin, Heidelberg, 2013.

[4] H. Pasch, Hyphenated Techniques in Liquid Chromatography of Polymers, Darmstadt, 1999.

[5] A.M. Skvortsov, A.A. Gorbunov, D. Berek, B. Trathnigg, Liquid chromatography of macromolecules at the critical adsorption point: Behaviour of a polymer chain inside pores, Polymer 39 (2) (1998) 423–429.

[6] D. Berek, Polymer HPLC, in: D. Corradini (Ed.), Handbook of HPLC, 2nd ed., Taylor & Francis, Boca Raton, FL, 2010, pp. 447–504.

[7] G. Glöckner, Polymercharakterisierung durch Flüssigkeits-Chromatographie: Chromatographische Methoden, Hüthig, Heidelberg, 1982.

[8] A.M. Striegel, W.W. Yau, J.J. Kirkland, D.D. Bly, Modern Size-Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography, 2nd ed., John Wiley & Sons, 2009.

[9] D. Berek, Size exclusion chromatography—a blessing and a curse of science and technology of synthetic polymers, J. Sep. Sci. 33 (3) (2010) 315–335.

[10] Y. Brun, Chromatography, HPLC, in: J.I. Kroschwitz, H.F. Mark (Eds.), Encyclopedia of Polymer Science and Technology, 3rd ed., Wiley-Interscience, Hoboken, NJ, 2003, pp. 598–613.

[11] G. Glöckner, Gradient HPLC of Copolymers and Chromatographic Cross-fractionation, Springer, Berlin, Heidelberg, New York, London, Paris, Tokyo, u.a, 1991.

[12] G. Glöckner, Polymer Characterization by Liquid Chromatography: Chromatographische Methoden, Elsevier, Amsterdam [u.a.], 1987.

[13] H. Philipsen, B. Klumperman, A.L. German, Characterization of low-molar-mass polymers by gradient polymer elution chromatography I. Practical parameters and applications of the analysis of polyester resins under reversed phase conditions, J. Chromatogr. A 746 (2) (1996) 211–224.

[14] W.J. Staal, Gradient Polymer Elution Chromatography: A Qualitative Study on the Prediction of Retention Times Using Cloud-points and Solubility Parameters, Technische Universiteit Eindhoven, Eindhoven, 1996.

[15] H. Philipsen, M.R. de Cooker, H.A. Claessens, B. Klumperman, A.L. German, Characterization of low-molar-mass polymers by gradient polymer elution chromatography II. Solubility effects in the analysis of polyester resins under reversed-phase conditions, J. Chromatogr. A 761 (1-2) (1997) 147–162.

[16] H. Philipsen, M. Oestreich, B. Klumperman, A. German, Characterization of low-molar-mass polymers by gradient polymer elution chromatography: III. Behaviour of crystalline polyesters under reversed-phase conditions, J. Chromatogr. A 775 (1-2) (1997) 157–177.

[17] Z. Deyl, K. Macek, J. Janák (Eds.), Liquid Column Chromatography: A Survey of Modern Techniques and Applications, Elsevier Scientific Pub. Co, Amsterdam, New York, 1975.

[18] P. Jandera, J. Churáček, Gradient Elution in Column Liquid Chromatography: Theory and Practice, Elsevier, Amsterdam [u.a.], 1985.

[19] P. Nikitas, A. Pappa-Louisi, K. Papachristos, Optimisation technique for stepwise gradient elution in reversed-phase liquid chromatography, J. Chromatogr. A 1033 (2) (2004) 283–289.

[20] P. Nikitas, A. Pappa-Louisi, New approach to linear gradient elution used for optimisation in reversed-phase liquid chromatography, J. Chromatogr. A 1068 (2) (2005) 279–287.

[21] P. Nikitas, A. Pappa-Louisi, P. Agrafiotou, Multilinear gradient elution optimisation in reversed-phase liquid chromatography using genetic algorithms, J. Chromatogr. A 1120 (1-2) (2006) 299–307.

[22] P. Nikitas, A. Pappa-Louisi, A. Papageorgiou, Simple algorithms for fitting and optimisation for multilinear gradient elution in reversed-phase liquid chromatography, J. Chromatogr. A 1157 (1-2) (2007) 178–186.

[23] S. Fasoula, C. Zisi, H. Gika, A. Pappa-Louisi, P. Nikitas, Retention prediction and separation optimization under multilinear gradient elution in liquid chromatography with Microsoft Excel macros, J. Chromatogr. A 1395 (2015) 109–115.

[24] J.W. Dolan, D.C. Lommen, L.R. Snyder, High-performance liquid chromatographic computer simulation based on a restricted multi-parameter approach: I. Theory and verification, J. Chromatogr. A 535 (1990) 55–74.

[25] P. Jandera, Simultaneous optimisation of gradient time, gradient shape and initial composition of the mobile phase in the high-performance liquid chromatography of homologous and oligomeric series, J. Chromatogr. A 845 (1-2) (1999) 133–144.

[26] M. Konáš, M. Kubín, Chromatographic separation of azeotropic and low-conversion poly(styrene-stat-2-methoxyethyl methacrylate) according to chemical composition, J. Appl. Polym. Sci. 47 (12) (1993) 2245–2255.

[27] S. Mori, Characterization of styrene-acrylonitrile copolymers by size exclusion Chromatography/Stepwise gradient elution—liquid precipitation chromatography, Int. J. Polym. Anal. Charact. 2 (2) (1996) 185–192.

[28] R. Góra, M. Hutta, M. Vrìka, S. Katuìcák, M. Jablonský, Characterization of Klason lignins by reversed-phase high-performance liquid chromatography using wide-pore octadecylsilica and stepwise gradients of dimethylformamide in water, J. Sep. Sci. 29 (14) (2006) 2179–2189.

[29] M. Hutta, R. Góra, Novel stepwise gradient reversed-phase liquid chromatography separations of humic substances, air particulate humic-like substances and lignins, J. Chromatogr. A 1012 (1) (2003) 67–79.

[30] A.C. Stenson, Reversed-phase chromatography fractionation tailored to mass spectral characterization of humic substances, Environ. Sci. Technol. 42 (6) (2008) 2060–2065.

[31] T. Kajdan, H. Cortes, K. Kuppannan, S.A. Young, Development of a comprehensive multidimensional liquid chromatography system with tandem mass spectrometry detection for detailed characterization of recombinant proteins, J. Chromatogr. A 1189 (1-2) (2008) 183–195.

[32] T. Spranger, D. van Pinxteren, H. Herrmann, Two-Dimensional Offline Chromatographic Fractionation for the Characterization of Humic-Like Substances in Atmospheric Aerosol Particles, Environ. Sci. Technol. 51 (9) (2017) 5061–5070.

[33] D.L. Morris, J.N. Sutton, R.G. Harper, A.T. Timperman, Reversed-phase HPLC separation of human serum employing a novel saw-tooth gradient: toward multidimensional proteome analysis, J. Proteome Res. 3 (6) (2004) 1149–1154.

[34] E. Maksimova, E. Vlakh, E. Sinitsyna, T. Tennikova, HPLC analysis of synthetic polymers on short monolithic columns, J. Sep. Sci. 36 (23) (2013) 3741–3749.

[35] B. Durner, T. Ehmann, F.-M. Matysik, Adaption of a parallel-path poly(tetrafluoroethylene) nebulizer to an evaporative light scattering detector: Optimization and application to studies of poly(dimethylsiloxane) oligomers as a model polymer, J. Chromatogr. A (2018).

[36] Dionex, Operational Qualification/ Performance Qualification for HPLC Instruments: Operating Instructions, 2008.

[37] R. Brereton, Applied Chemometrics for Scientists, John Wiley & Sons, Chichester, 2007.

[38] W. Kleppmann, Versuchsplanung: Produkte und Prozesse optimieren, 9th ed., Hanser, München, 2016.

[39] T. Mori (Ed.), Taguchi Methods: Benefits, Impacts, Mathematics, Statistics, and Applications, American Society of Mechanical Engineers, New York, N.Y. (ASME, Three Park Avenue. New York, NY 10016), 2011.

[40] G. Taguchi, S. Chowdhury, Y. Wu, S. Taguchi, H. Yano (Eds.), Taguchi's Quality Engineering Handbook, John Wiley & Sons, Hoboken, N.J, Livonia, Mich, 2011.

[41] D.M. Byrne, S. Taguchi, The Taguchi approach to parameter design, in: ASQC Conference Proceedings, 1986.

[42] L. Li (Ed.), MALDI Mass Spectrometry for Synthetic Polymer Analysis, Wiley, Hoboken, N.J, 2010.