Journal of Chromatography A, 1222 (2012) 71–80

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A general strategy for performing temperature-programming in high performance liquid chromatography—Further improvements in the accuracy of retention time predictions of segmented temperature gradients

Steffen Wiese[a,b], Thorsten Teutenberg[a,*], Torsten C. Schmidt[b]

[a] Institut für Energie- und Umwelttechnik e. V., Bliersheimer Strasse 58-60, 47229 Duisburg, Germany
[b] University Duisburg-Essen, Instrumental Analytical Chemistry, Universitätsstrasse 5, 45141 Essen, Germany

ARTICLE INFO

Article history:
Received 17 August 2011
Received in revised form 17 October 2011
Accepted 5 December 2011
Available online 13 December 2011

Keywords:
Temperature-programming
High-temperature high performance liquid chromatography (HT-HPLC)
Heating system
Method development
Linear elution strength model (LES)
Linear solvent strength model (LSS)

ABSTRACT

In the present work it is shown that the linear elution strength (LES) model which was adapted from temperature-programming gas chromatography (GC) can also be employed for systematic method development in high-temperature liquid chromatography (HT-HPLC). The ability to predict isothermal retention times based on temperature-gradient as well as isothermal input data was investigated. For a small temperature interval of $\Delta T = 40\,^{\circ}C$, both approaches result in very similar predictions. Average relative errors of predicted retention times of 2.7% and 1.9% were observed for simulations based on isothermal and temperature-gradient measurements, respectively. Concurrently, it was investigated whether the accuracy of retention time predictions of segmented temperature gradients can be further improved by temperature dependent calculation of the parameter S_T of the LES relationship. It was found that the accuracy of retention time predictions of multi-step temperature gradients can be improved to around 1.5%, if S_T was also calculated temperature dependent. The adjusted experimental design making use of four temperature-gradient measurements was applied for systematic method development of selected food additives by high-temperature liquid chromatography. Method development was performed within a temperature interval from 40 °C to 180 °C using water as mobile phase. Two separation methods were established where selected food additives were baseline separated. In addition, a good agreement between simulation and experiment was observed, because an average relative error of predicted retention times of complex segmented temperature gradients less than 5% was observed. Finally, a schedule of recommendations to assist the practitioner during systematic method development in high-temperature liquid chromatography was established.

1. Introduction

The use of elevated temperature in high-performance liquid chromatography (HPLC) is not a new topic of investigation [1] and it is well documented that increasing the temperature results in a change of the physicochemical properties of water and binary solvent mixtures [2–4]. However, the parameter temperature enables some special hyphenation techniques [5–12]. Most of these techniques use the decrease in the static permittivity of water at elevated temperatures [4]. In other words, the higher the temperature of water, the lower the polarity of a water mobile phase. Hence, under certain conditions temperature gradients can be employed instead of solvent gradients, which has been shown elsewhere [13–16]. Consequently, the user is faced with the problem to develop a method where temperature gradients are employed instead of solvent gradients.

For method development in solvent gradient elution, several software packages like DryLab [17], ChromSwordAuto [18], Osiris [19] or ACD/LC & GC Simulator [20] are commercially available to assist the user and to reduce the necessary experimental efforts. Unfortunately, these software packages do not permit the simulation of the retention time of an analyte depending on a temperature gradient due to the lack of a suitable retention model. In other words, most attempts to achieve good separations in temperature-programming mode are governed by trial and error [21–24]. This problem was first recognized by Nikitas and Pappa-Louisi. They developed retention models which permit prediction of retention times when solvent composition and temperature are changed simultaneously [25,26]. Up to now, their models were tested using only linear temperature gradients with moderate slopes from 2 °C min^{-1} up to 10 °C min^{-1} in a temperature interval from

* Corresponding author. Tel.: +49 0 2065 418 179, fax: +49 0 2065 418 211.
E-mail address: teutenberg@iuta.de (T. Teutenberg).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.12.018

15 °C up to 75 °C ($\Delta T = 60$ °C). Recently, Cela and co-workers have described computer-assisted method development in high temperature liquid chromatography based on an evolutionary algorithm [27]. The developed approach also permits dual mode predictions of retention times when solvent composition and temperature are changed simultaneously. During their study a temperature interval from 40 °C to 180 °C was investigated using temperature-gradient slopes up to 20 °C min^{-1}. Moreover, they noted that their software package PREGA has incorporated this methodology and can be downloaded for free [27].

In a recent study [28] we could show that the linear elution strength (LES) model from temperature-programmed gas chromatography (GC) can be employed for predictions of linear temperature gradients in temperature-programmed liquid chromatography. The high accuracy of retention time predictions was shown for selected steroids and polycyclic aromatic hydrocarbons (PAHs) in a temperature interval from 50 °C up to 180 °C when temperature gradients with slopes up to 30 °C min^{-1} were applied. In a further study [29] the LES model was extended in order to predict more complex segmented temperature gradients in a similar temperature interval (60–180 °C). It was concluded that the accuracy of retention time predictions was lower if the start temperature of the predicted gradient was not equal to the start temperature of the measurements which have been employed during data fitting.

Moreover, systematic method development in liquid chromatography should be performed using as few input measurements as possible. In order to reduce the experimental work it would be advantageous if isothermal as well as temperature-gradient simulations can be performed based only on temperature-gradient data. Data acquisition using temperature-gradient measurements is less time consuming when compared to isothermal data acquisition. Furthermore, samples containing analytes with different polarities can be measured within the same chromatographic run in temperature-gradient mode. If isothermal data are required, long analysis times are expected for the less polar compounds of the sample mixture at low temperature.

Therefore, this study investigated the ability to predict segmented temperature-gradients based on only temperature-gradient input measurements. Concurrently it will be explored whether the accuracy of retention time predictions of complex segmented temperature-gradients can be improved using a new experimental design as well as a temperature dependent calculation of the parameter S_T of the LES model. In addition, the applicability of systematic temperature-programming method development by means of the LES model will be investigated using as few input measurements as possible. For this reason, several methods will be developed for the separation of selected food additives using a water mobile phase. Finally, a schedule of recommendations will be given to assist the user during systematic temperature-programming method development in high-temperature liquid chromatography.

2. Experimental

2.1. Chemicals

High-purity deionized water was prepared by an Elix 10-Milli-Q Plus water purification system (Millipore, Eschborn, Germany). Acetonitrile (Optigrade) was purchased from LGC Standards (Wesel, Germany). In this study a mixture of six food additives was employed including theobromine, theophylline, catechine, caffeine, aspartame, rutin, and uracil. All chemicals employed in this study except for the solvents were purchased from Sigma–Aldrich (Seelze, Germany) and were of p.a. grade. Stock solutions were prepared by dissolving an equivalent amount of the analytes in water to obtain a concentration of 1.0 mg mL^{-1} of theophylline, catechine and aspartame. Uracil, theobromine, caffeine, and rutin were dissolved in a mixture of 50/50 (v/v) water/acetonitrile at a concentration of 0.5 mg mL^{-1}. 0.1% formic acid was added to adjust the pH of the stock solutions to 2.7.

2.2. HPLC system

A Shimadzu HPLC system (Shimadzu, Duisburg, Germany) was used consisting of two LC-10AD$_{VP}$ pumps, a DGU-14 A degasser, an SIL-10AD$_{VP}$ autosampler, an SPD-M10A$_{VP}$ diode array detector (DAD), and an SCL-10A$_{VP}$ controller. A 500 psi backpressure regulator (GammaAnalysenTechnik, Bremerhaven, Germany) was connected behind the DAD to keep the mobile phase in the liquid state. For data acquisition and analysis, Shimadzu LCsolution (version 1.21 SP 1) was used. All measurements in the present study were carried out on a Waters XBridge C-18 (50 mm × 3.0 mm, 3.5 μm) column at a flow rate of 0.5 mL min^{-1} using a water mobile phase with 0.1% formic acid. This column was chosen because of its very good temperature and pH stability [30]. UV detection was performed at a wavelength of 200 nm.

2.3. Heating system

To heat the mobile and stationary phase a commercially available SIM HT-HPLC 200 high-temperature column oven (SIM – Scientific Instruments Manufacturer, Oberhausen, Germany) was used [31,32]. The heating system was designed for high-temperature liquid chromatography and consists of three modules: the eluent preheating unit, the column heating unit and the eluent cooling unit. The heat transfer is achieved by block heating which means that the capillaries and column are tightly enclosed by aluminium blocks. The three heating units can be controlled independently, which guarantees that the temperature of the mobile phase entering the column and the temperature of the stationary phase can be exactly matched. If a temperature gradient is applied, the temperature of the preheating unit and the temperature of the column are increased simultaneously. For all measurements performed in this study, the temperature setting of the preheating unit and the column were identical.

2.4. Isothermal/isocratic measurements

For the isothermal measurements under isocratic conditions, three test mixtures were employed. The first mixture was composed of theobromine, theophylline and aspartame. The second mixture included catechine and caffeine. Rutin was measured separately. The concentration of each food additive was set to 0.1 mg mL^{-1} in each mixture and uracil was added to yield a final concentration of 0.01 mg mL^{-1}. The investigated temperature interval ranged from 40 °C to 120 °C with increments of 10 °C, except for rutin where a temperature interval from 90 °C to 120 °C was investigated.

2.5. Temperature-gradient measurements

For these measurements a mixture of all food additives was prepared by adding an equivalent amount of each stock solution to obtain a concentration of 0.1 mg mL^{-1} of each analyte in the mixture. Uracil was added to obtain a final concentration of 0.01 mg mL^{-1}. The start temperature of the temperature gradients ranged from 40 °C to 80 °C with increments of 10 °C. The temperature difference ΔT ($\Delta T = T_{final} - T_{start}$) between start and final temperature was set to 100 °C and gradient slopes of 2, 4, 6 and

Table 1
Schedule of the experimental temperature gradients which were employed as input runs.

Run number	Gradient slope (°C min^{-1})	Start temperature (°C)	Final temperature (°C)
1	2	40	140
2	4		
3	6		
4	8		
5	2	50	150
6	4		
7	6		
8	8		
9	2	60	160
10	4		
11	6		
12	8		
13	2	70	170
14	4		
15	6		
16	8		
17	2	80	180
18	4		
19	6		
20	8		

8 °C min^{-1} were applied. Table 1 summarizes the temperature-gradient measurements which have been employed as input data.

3. Theory

In a previous study [28] we have shown that the LES model could successfully be adapted from temperature-programmed gas chromatography to temperature-programmed liquid chromatography. Furthermore, it was shown that it was not necessary to extend the LES model to consider a temperature-dependent delay time when a high-temperature column oven based on block heating was employed. Using the LES model the retention time t_R of an analyte can be predicted as a function of experimental conditions using Eqs. (1) and (2) [33,34].

$$t_R = \frac{t_0}{2.3 b_T} \ln[e^{2.3 b_T}(k_0 + 1) - k_0] \quad (1)$$

with

$$b_T = \frac{t_0 S_T \Delta T}{tG} \quad (2)$$

where t_0 is the column dead time and k_0 is the retention factor of the solute at the start of the temperature gradient that should theoretically equal the retention factor obtained in isothermal conditions. The temperature gradient-steepness parameter b_T consists of the solute constant S_T, the temperature range ΔT ($\Delta T = T_{final} - T_{start}$) and the temperature gradient time tG. For the prediction of retention times, two experimental temperature-gradient runs are required. These runs should differ in temperature-gradient slopes by a factor of at least three whereas all other experimental conditions are kept constant [33,35]. Moreover, for reliable predictions the analytes should elute within the temperature-gradient window. On the basis of two temperature-gradient measurements, values of S_T and k_0 for each analyte are derived by numerical solution of Eqs. (1) and (2). This procedure is very similar to numerical solutions of the LSS relationship [36–38].

In order to predict retention times for segmented temperature gradients, an equation is required which describes the fractional migration r of the solute across the column during a given temperature segment. In other words, r is the distance in longitudinal direction which an analyte moves through the column during a temperature segment. In this case, a similar derivation to solvent gradient elution yields Eq. (3) [33,39,40].

$$t_R = \frac{t_0}{2.3 b_T} \ln[e^{2.3 b_T r}(k_0 + 1) - k_0] \quad (3)$$

Furthermore, in the case where a temperature-gradient method consists of an isothermal/isocratic hold, Eq. (4) can be employed to calculate the retention time of an analyte during this segment.

$$t_R = r t_0 (k_0 + 1) \quad (4)$$

The sum of the fractional migration r of an analyte across the column during each temperature segment can be written as ($r_1 + r_2 + \cdots r_n = 1$). Moreover, Eq. (5) describes the change of the retention factor of an analyte during each temperature segment and is required to calculate the retention factor k_r of the analyte at the end of a temperature segment. This value has then to be used as initial value for the next temperature segment and is employed instead of k_0 in Eq. (3).

$$\log k_r = \log k_0 - \frac{b_T t_R}{t_0} \quad (5)$$

Finally, the sum of the calculated retention times of an analyte for all temperature segments represents the total retention time of a multi-step temperature gradient. An example of how a spreadsheet calculator can be used to calculate the total retention time of an analyte depending on segmented temperature gradients is given in the Supporting Information.

Recently, it was shown that a plot of $\ln k$ vs. T can be employed for isothermal retention time predictions [29], and a combination of isothermal and temperature-gradient input measurements has been employed to predict temperature gradients with different start temperatures. Nevertheless, the accuracy of retention time predictions based on two temperature gradients and two isothermal runs was inferior to the accuracy of predictions where the start temperature of the gradient was equal to the start temperature of the input runs. It was concluded that both LES parameters k_0 and S_T should be calculated temperature dependent [29]. For this approach it is necessary to change the experimental design of the input measurements. To that end, four temperature-gradient measurements were carried out, with two runs at a low start temperature of, e.g., 40 °C, and two runs at a higher temperature of, e.g., 80 °C, keeping all other experimental conditions constant (ΔT, tG). Afterwards, the LES parameters k_0 and S_T were calculated for the lower and the higher start temperature. To calculate the parameter S_T depending on temperature a linear regression of a plot of S_T vs. T has been employed for interpolation. Similar to the parameter S_T the parameter k_0 can also be calculated depending on temperature by means of linear regression of an $\ln k_0$ vs. T plot [29].

4. Results and discussion

4.1. Isothermal predictions based on isothermal and temperature-gradient input data

In the present work, the experimental design as presented in a previous study [29] was changed to investigate the ability to express the influence of temperature on the retention factor of a solute based on four temperature-gradient runs. To test this approach a data set of ten temperature-gradient measurements was employed, where the start temperature of the gradients ranged from 40 °C to 80 °C. The gradient slopes were set to 2 °C min^{-1} and 6 °C min^{-1} at each start temperature (runs 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19 of Table 1). On the basis of these runs values of k_0 for each analyte were calculated depending on the start temperature by means of the approach described in the theoretical section (Eqs. (1) and (2)). Afterwards, the calculated values of k_0 were employed

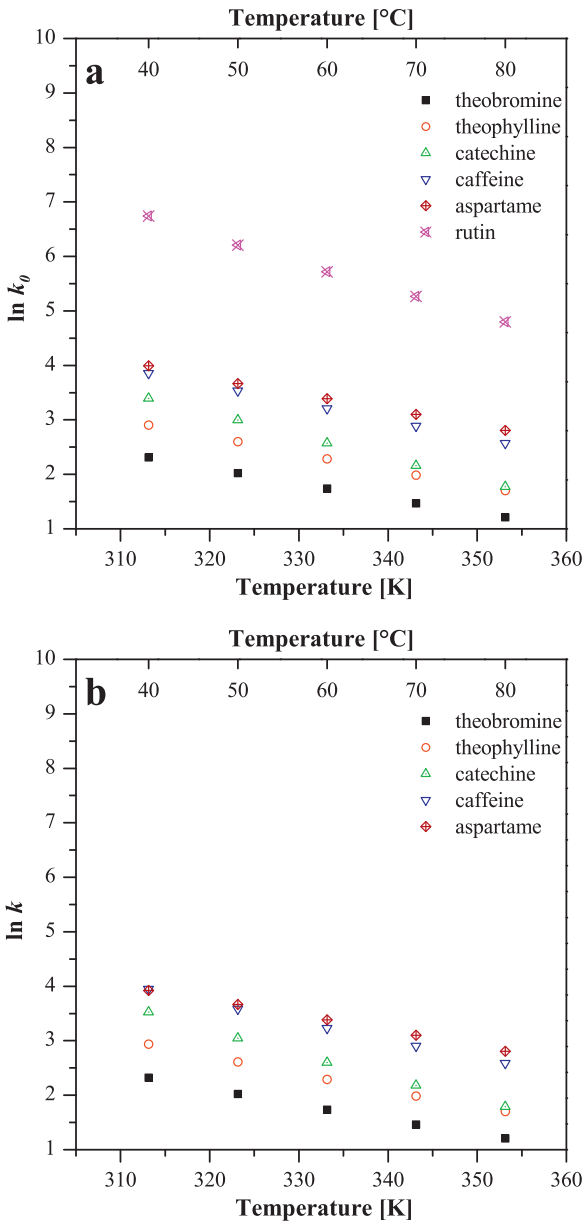

Fig. 1. Comparison of different plots of ln k vs. T. (a) calculated values of ln k_0 based on temperature gradients, (b) calculated values of ln k based on isothermal measurements. Chromatographic conditions: stationary phase: Waters XBridge C-18 (50 mm × 3.0 mm, 3.5 μm); mobile phase: water + 0.1% formic acid; injection volume: 4 μL, see also Sections 2.4 and 2.5.

to represent a plot of ln k_0 vs. T which is shown in Fig. 1a. The corresponding plot of ln k vs. T based on isothermal measurements is shown in Fig. 1b.

As can be seen, both plots look very similar and show a strict linear relationship between the natural logarithm of the retention factor vs. temperature for each food additive. This is confirmed by the data given in Table 2 where characteristics of a linear regression for both plots are represented. The values for the slopes and intercepts of the linear equations were comparable for all plots. Moreover, the linear behavior was underlined by the coefficients of correlation (R^2) ranging between 0.9973 and 0.9999, regardless of performing the regression with isothermal or temperature-gradient input data.

Moreover, Fig. 1a underlines that an advantage of isothermal retention time predictions based on temperature gradients is the ability to predict isothermal retention times of rutin at a temperature below 90 °C. Isothermal data acquisition for rutin at a temperature below 90 °C is not reasonable, because very long analysis times will be expected. For example, if the measurements are carried out at a temperature of 40 °C rutin needs approximately 6 h to elute from the column. Furthermore, the high linear relationship of the plots of ln k_0 vs. T as well as ln k vs. T allows the prediction of isothermal retention times using only experimental data at two temperatures. In order to compare the accuracy of isothermal retention time predictions based on temperature-gradient as well as isothermal measurements, Table 3 reveals a comparison of relative errors of interpolated (50–70 °C) and extrapolated (90–120 °C) isothermal retention times of selected food additives.

In order to compare relative errors, in a first step isothermal retention time calculations were performed by linear regression using isothermal data at 40 °C and 80 °C. For the retention time calculations based on temperature gradients, two gradient runs within a temperature interval from 40 °C to 140 °C with gradient slopes of 2 °C min^{-1} and 6 °C min^{-1} (runs 1 and 3 of Table 1) as well as two runs within a temperature interval from 80 °C to 180 °C with the same slopes (runs 17 and 19 of Table 1) were employed. Afterwards, values of k_0 were calculated corresponding to the start temperature of the basic measurements (40 °C and 80 °C). Subsequently a linear regression of ln k_0 vs. T was performed in order to calculate isothermal retention times based on temperature-gradient data. Finally, relative errors were calculated by a comparison of predicted and experimental retention times of the food additives (Table 3).

It can be seen that the relative errors of interpolated isothermal retention times based on isothermal data and temperature gradients are very similar. An average relative error of 2.7% and 1.9% was calculated for isothermal and temperature-gradient input data, respectively. In the case of extrapolations to higher temperatures, e.g., 120 °C, larger differences between predicted and experimental retention times are observed. Regarding our measurements it can be pointed out that extrapolations based on both isothermal and temperature-gradient data, should not exceed a temperature of 100 °C corresponding to an extrapolation limit of 25%. Otherwise, major relative errors up to 21% of predicted retention times would be observed. The results shown in Table 3 underline that isothermal predictions based on isothermal input data should only be applied for a small temperature interval of e.g., $\Delta T = 40$ °C when using only two temperatures for data fitting. To predict isothermal retention times using a larger temperature interval of e.g., $\Delta T = 100$ °C, at least data at three temperatures should be employed to describe the influence of temperature on retention. In this context, isothermal retention time predictions based on temperature-gradient data can be a helpful and time saving tool, in order to get information whether an isothermal separation of selected analytes is possible. In addition, this kind of predictions can be employed for the design of experiments of isothermal measurements. A detailed discussion regarding an isothermal separation of selected food additives is given in the Supporting Information.

4.2. Temperature-gradient predictions based on gradient input data

The main aim of our efforts regarding isothermal retention time predictions based on temperature-gradient measurements was to investigate the suitability of four temperature-gradient runs to predict retention times for other temperature gradients with a different start temperature. The idea was to use two temperature gradients with a start temperature of 40 °C and two runs with a start temperature of 80 °C to predict other temperature gradients with a start temperature between 40 °C and 80 °C. Concurrently, it was investigated whether the accuracy of these predictions could be improved by temperature dependent fitting of the LES parameter S_T. In other words, for the temperature dependent calculation of

Table 2
Overview of characteristics of linear regression of the plots shown in Fig. 1.

Figure	Parameter	Theobromine	Theophylline	Catechine	Caffeine	Aspartame	Rutin
1a	Slope	-2.75×10^{-2}	-3.02×10^{-2}	-4.08×10^{-2}	-3.21×10^{-2}	-2.94×10^{-2}	-4.83×10^{-2}
	Intercept	1.09×10^{1}	1.24×10^{1}	1.62×10^{1}	1.39×10^{1}	1.32×10^{1}	2.18×10^{1}
	R^2	0.9994	0.9997	0.9998	0.9999	0.9993	0.9989
1b	Slope	-2.82×10^{-2}	-3.03×10^{-2}	-4.24×10^{-2}	-3.20×10^{-2}	-2.61×10^{-2}	–
	Intercept	1.06×10^{1}	1.19×10^{1}	1.62×10^{1}	1.34×10^{1}	1.16×10^{1}	–
	R^2	0.9973	0.9981	0.9989	0.9991	0.9998	–

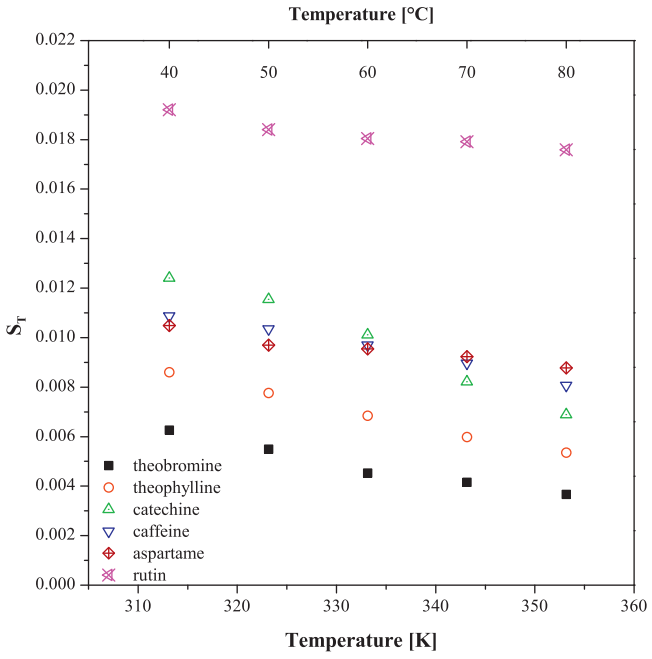

Fig. 2. Plot of S_T vs. T of six food additives. Calculated values of S_T based on experimental temperature-gradient measurements. For chromatographic conditions see Section 2.

S_T the same temperature-gradient data set was used that has been employed for the temperature dependent calculation of k_0 which was described in Section 4.1 (runs 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19 of Table 1). On the basis of these measurements, values of S_T for each analyte were calculated depending on the start temperature by means of the approach described in the theoretical section (Eqs. (1) and (2)). Afterwards, the calculated values of S_T were employed to plot S_T vs. T which is shown in Fig. 2.

Fig. 2 points out that the parameter S_T decreases with increasing start temperature of the gradients which have been employed during data fitting. Moreover, the variation of S_T depending on the start temperature can be described by a linear relationship. This can also be seen in Table 4 where characteristics of the linear regression of the S_T vs. T plot are represented for each food additive.

The coefficients of correlation (R^2) for the food additives are satisfactory except for rutin where a less linear relationship between S_T and T was observed. Nevertheless, based on the data given in Table 4 it is acceptable to calculate the parameter S_T depending on temperature when using only experimental data at two start temperatures, in order to increase the accuracy of retention time predictions.

To test this approach, two temperature gradients with a start temperature of 40 °C and two runs with a start temperature of 80 °C (runs 1, 3, 17, and 19 of Table 1) were employed to calculate S_T depending on temperature by means of linear regression of the S_T vs. T plot. Afterwards, the retention times of selected food additives were predicted for a set of twelve temperature gradients where different start temperatures from 50 °C to 70 °C and different gradient slopes from 2 °C min^{-1} up to 8 °C min^{-1} were applied. Predicted and experimental data are compared in Table 5. In the case where the retention times of the food additives were not calculated based on temperature dependent fitting of S_T, the required values of S_T were taken from data fitting at a temperature of 40 °C. The relative errors of these predictions are also shown in Table 5.

When the start temperature of the temperature gradient was increased from 40 °C up to 50 °C, it was not necessary to calculate S_T depending on temperature, because a maximal relative error of predicted retention times of 3.9% was obtained. When the start temperature was increased further to 60 °C, a larger maximal relative error of 5.9% of predicted retention times of the food additives was calculated. Considering the average relative error of predicted retention times of all food additives, it can be concluded that a temperature dependent calculation of both parameters S_T and k_0 results in an average relative error of 0.9% whereas, if only the parameter k_0 was calculated depending on temperature, an average relative error of 2.4% was observed. In other words, if both parameters are calculated depending on temperature the accuracy of predicted retention times can be increased to around 1.5%. Hence, S_T as well as k_0 should be calculated temperature-dependent in order to obtain more reliable retention time predictions.

Because of the results shown in Fig. 1 and Table 5, it is possible to use four temperature-gradient runs during systematic method development in LC instead of two temperature-gradient runs and two isothermal measurements which were employed during a previous study [29].

4.3. Systematic temperature-programming method development

The new experimental design has been employed to perform systematic temperature-programming method development of

Table 3
Comparison of relative errors of isothermal retention time predictions based on isothermal and on temperature gradient measurements. Roman character corresponds to isothermal measurements. *Italic* character corresponds to temperature-gradient measurements.

Predicted temperature (°C)	Theobromine (%)		Theophylline (%)		Catechine (%)		Caffeine (%)		Aspartame (%)	
50	2.0	*1.3*	1.9	*0.3*	4.1	*5.4*	2.6	*4.5*	1.8	*3.3*
60	2.6	*2.3*	2.7	*1.2*	5.6	*1.3*	3.5	*1.7*	1.6	*1.7*
70	2.1	*2.2*	2.3	*1.6*	4.0	*0.2*	2.4	*0.7*	1.3	*0.2*
90	2.6	*1.9*	2.8	*2.2*	5.2	*4.4*	3.4	*2.7*	2.1	*0.4*
100	5.5	*4.7*	6.0	*4.9*	10.5	*8.1*	7.5	*5.2*	3.0	*0.4*
110	9.5	*8.5*	10.2	*8.7*	16.4	*12.9*	12.3	*8.6*	4.9	*0.1*
120	12.9	*11.9*	14.1	*12.4*	21.4	*17.5*	17.3	*12.8*	6.4	*0.1*

Table 4
Overview of characteristics of linear regression of the S_T vs. T plot for each food additive. Data shown here correspond to Fig. 2.

Parameter	Theobromine	Theophylline	Catechine	Caffeine	Aspartame	Rutin
Slope	-6.55×10^{-5}	-8.27×10^{-5}	-1.44×10^{-4}	-7.00×10^{-5}	-3.89×10^{-5}	-3.72×10^{-5}
Intercept	2.66×10^{-2}	3.45×10^{-2}	5.78×10^{-2}	3.29×10^{-2}	2.25×10^{-2}	3.06×10^{-2}
R^2	0.9720	0.9960	0.9864	0.9899	0.9462	0.9070

selected food additives by high-temperature liquid chromatography using a water mobile phase. As basic input data the following experimental temperature-gradient measurements were employed:

- 40–140 °C with a slope of 2 °C min^{-1} (run 1 of Table 1)
- 40–140 °C with a slope of 6 °C min^{-1} (run 3 of Table 1)
- 80–180 °C with a slope of 2 °C min^{-1} (run 17 of Table 1)
- 80–180 °C with a slope of 6 °C min^{-1} (run 19 of Table 1)

On the basis of these runs, several methods were developed with the aim to achieve a baseline separation of selected food additives. In other words, the critical resolution (R_S) should be higher than 1.5. In this context it has to be considered that method development is still based on trial and error and an optimization algorithm will be required. However, the development of such an algorithm is beyond the scope of this study. Here we would like to emphasize that retention time predictions can be performed using four basic temperature-gradient measurements. This will be discussed by the temperature-gradient methods shown in Fig. 3, where the start temperature for the gradients ranged from 40 °C to 70 °C.

Moreover, in Table 6 predicted and experimental retention times, relative errors and average relative errors are compared. In the case of a simple linear temperature gradient (Fig. 3b) the retention times can be predicted precisely, because a maximal relative error of 4.3% was observed and an average relative error of 2.6% was calculated. In the case of more complex segmented temperature gradients which are shown in Fig. 3a, c and d it can be concluded that the accuracy of predicted retention times decreases with increasing number of temperature segments during the separation. For example, theophylline and catechine elute in every separation shown in Fig. 3 during the first segment of the temperature gradient and the relative error ranges between 1.0% and 3.5%. In contrast, rutin usually elutes during the third (Fig. 3a and d) or fourth (Fig. 3c) segment of the temperature gradient and shows a slightly larger relative error between 3.5% and 5.6%. In comparison to that, if a simple linear temperature gradient is considered (Fig. 3b) a significantly lower relative error of predicted retention time of 1.1% is observed for rutin.

Regarding the aim to develop a temperature-gradient method which separates the food additives with a critical resolution higher than 1.5, only two methods were found to be suitable. At a start temperature of the gradient of 50 °C (Fig. 3b) the food additives can be separated within approximately 14 min with a critical resolution between peak pair 5/6 of 1.51. If the start temperature of the gradient was increased to 70 °C and a multi-step temperature gradient was applied (Fig. 3d), the analytes were separated within 9 min with a critical resolution between peaks 5/6 of 1.61. The critical resolution between peaks 5/6 of the methods shown in Fig. 3a and c were 1.36 and 1.49, respectively. In order to improve the critical resolution further, it would be feasible to double the column length. In this case the resolution of the method shown in Fig. 3d should increase from 1.61 to 2.27, but it has to be considered that the analysis time would also be doubled.

4.4. Repeatability and robustness of a temperature-gradient method

A prerequisite for a successful implementation of a temperature-gradient method in routine laboratory practice is the repeatability as well as robustness of an HPLC method. In order to investigate the repeatability and robustness at very high temperature as well as using moderate and high temperature-gradient slopes of the column oven, another method was chosen than suggested in Section 4.3 (Fig. 3d). The start temperature and the final temperature of the method were set to 50 °C and 180 °C, respectively. The temperature-gradient method consists of three segments, two gradients with slopes of 7.5 °C min^{-1} and 31.9 °C min^{-1} as well as an isothermal hold at 180 °C.

Fig. 4 shows an overlay of nine consecutive chromatograms of the separation of six food additives.

As can be seen, there are only marginal differences between the nine chromatograms which is also underlined by the statistical data given in Table 7.

The standard deviation of the retention times of the food additives ranged between 0.01 min and 0.02 min which corresponds to a relative standard deviation (RSD) between 0.05% and 0.23%. These values are comparable to the relative standard deviation of retention times obtained for conventional solvent gradient

Table 5
Comparison of relative errors of predicted retention times of food additives based on temperature gradient measurements. Roman character corresponds to temperature dependent fit of k_0 and S_T. *Italic* character indicates that only the parameter k_0 was fitted temperature dependent.

Temperature range (°C)	Slope (°C min^{-1})	Theobromine (%)		Theophylline (%)		Catechine (%)		Caffeine (%)		Aspartame (%)		Rutin (%)	
50–150	2	1.0	*0.5*	0.6	*0.3*	0.0	*1.9*	0.4	*1.1*	1.4	*0.4*	0.0	*1.4*
	4	0.5	*0.3*	0.4	*1.1*	0.9	*2.1*	0.9	*1.4*	0.8	*0.7*	0.4	*1.2*
	6	0.8	*0.4*	0.5	*1.6*	0.9	*2.9*	0.7	*2.0*	0.2	*1.5*	0.5	*2.1*
	8	0.7	*0.8*	0.2	*2.3*	0.4	*3.9*	0.0	*2.9*	0.4	*2.3*	1.6	*3.2*
60–160	2	1.8	*1.1*	1.4	*0.0*	1.4	*1.5*	1.1	*1.4*	0.7	*1.0*	0.8	*1.9*
	4	1.8	*0.5*	1.3	*1.2*	2.1	*2.7*	1.8	*2.0*	0.6	*1.9*	1.3	*1.7*
	6	1.3	*0.6*	1.1	*2.3*	1.9	*4.3*	1.3	*3.3*	0.3	*2.8*	0.1	*3.0*
	8	1.3	*1.1*	0.7	*3.4*	1.3	*5.9*	0.6	*4.6*	0.3	*3.7*	1.2	*4.3*
70–170	2	1.4	*0.5*	1.4	*0.2*	1.4	*1.7*	1.0	*1.9*	0.2	*1.8*	0.4	*3.3*
	4	1.5	*0.1*	1.2	*1.8*	1.6	*3.8*	1.4	*3.3*	0.5	*2.7*	1.3	*2.9*
	6	1.2	*1.1*	1.1	*3.0*	1.2	*6.0*	1.2	*4.8*	0.3	*3.8*	0.2	*4.2*
	8	0.5	*2.4*	0.1	*5.0*	0.3	*8.2*	0.3	*6.5*	0.5	*5.0*	1.4	*5.8*
Average error (%)		1.2	*0.8*	0.8	*1.9*	1.1	*3.7*	0.9	*2.9*	0.5	*2.3*	0.8	*2.9*

Table 6
Comparison of predicted retention times (pred. RT) calculated by LES model and experimental retention times (expt. RT) of selected food additives. Data shown here correspond to Fig. 3.

Figure	Analytes	Seg. [a]	Peak width (min)[b]	Expt. RT (min)	Pred. RT (min)	Difference (min)	Relative error (%)	Average rel. error (%)
3a	Theobromine	1	0.29	3.62	3.73	0.12	3.2	2.8
	Theophylline	1	0.30	5.01	5.07	0.06	1.2	
	Catechine	1	0.22	5.81	5.70	0.11	1.9	
	Caffeine	2	0.34	7.56	7.67	0.11	1.4	
	Aspartame	2	0.44	8.07	8.50	0.43	5.3	
	Rutin	3	0.19	13.35	12.88	0.47	3.5	
3b	Theobromine	1	0.28	3.10	3.23	0.13	4.3	2.6
	Theophylline	1	0.32	4.47	4.61	0.14	3.1	
	Catechine	1	0.25	5.27	5.33	0.06	1.2	
	Caffeine	1	0.39	7.30	7.48	0.19	2.5	
	Aspartame	1	0.46	7.91	8.18	0.27	3.5	
	Rutin	1	0.25	13.39	13.24	0.15	1.1	
3c	Theobromine	1	0.27	2.66	2.75	0.09	3.3	2.7
	Theophylline	1	0.35	3.98	4.08	0.10	2.6	
	Catechine	1	0.29	4.72	4.78	0.06	1.2	
	Caffeine	2	0.42	7.16	7.07	0.09	1.2	
	Aspartame	2	0.42	7.75	7.59	0.16	2.1	
	Rutin	4	0.15	10.28	9.70	0.58	5.6	
3d	Theobromine	1	0.23	2.16	2.25	0.09	4.2	3.0
	Theophylline	1	0.30	3.22	3.33	0.11	3.5	
	Catechine	1	0.27	3.69	3.73	0.04	1.0	
	Caffeine	2	0.33	5.92	5.81	0.11	1.9	
	Aspartame	2	0.31	6.41	6.28	0.13	2.0	
	Rutin	3	0.17	8.97	8.50	0.46	5.2	

[a] The elution of the analyte was carried out during the denoted temperature segment.
[b] The peak width was calculated at 10% peak height.

Table 7
Overview of statistical data of nine consecutive chromatograms for the separation of six food additives. Data shown correspond to Fig. 4.

Parameter	Theobromine	Theophylline	Catechine	Caffeine	Aspartame	Rutin
Retention time (min)	3.05	4.44	5.31	7.28	7.77	12.59
Standard deviation (min)	0.01	0.01	0.01	0.01	0.02	0.01
Relative standard deviation (RSD) (%)	0.19	0.18	0.15	0.14	0.23	0.05

elution. Moreover, these results underline that even complex temperature gradients with high gradient slopes lead to very low deviations in retention time predictions for all analytes. Furthermore, the high repeatability of complex temperature-gradient measurements allows the conclusion that the rather simple linear basic input temperature-gradient runs will have only a minor contribution to the error observed for predicted retention times in temperature-programming mode (see Section 4.3). In other words, the obtained relative error of predicted retention times is related to the retention model and not to the input measurements which have been employed for data fitting.

Another important prerequisite for the successful implementation of a temperature-gradient method in routine laboratory practice is the robustness of the separation method. Here, the robustness will be discussed in terms of the critical resolution using the same method which has been employed for the evaluation of the repeatability. Table 8 shows a comparison of the critical resolution between caffeine and aspartame when the temperatures of the gradient points were changed by ±2 °C.

As can be seen, decreasing the temperature of the gradient points by −2 °C results in a decrease of the critical resolution from 1.13 to 1.06. In the case when the temperature of the gradient points was increased by 1 °C the critical resolution also decreases from 1.13 to 1.09, but when increasing the temperature further to +2 °C an increase of the critical resolution was observed. The results given in Table 8 underline that the critical resolution will be affected even by small changes of the temperature of the gradient points. Moreover, it can be assumed that similar changes will be observed if the time of the temperature-gradient points will be changed slightly (chromatograms regarding this topic are given in the Supporting Information).

Regarding the temperature-gradient method preferred for the separation of the food additives discussed in Section 4.3 (Fig. 3d), it can be concluded that the method is less robust especially when the temperature as well as the time of the gradient points will be changed even slightly. In order to avoid issues regarding the critical resolution in routine laboratory practice, the column length should be increased to achieve a higher critical resolution.

Table 8
Change of the critical resolution between caffeine and aspartame when varying the temperature of the gradient points based on the method depicted in Fig. 4.

	$\Delta T = -2$ °C		$\Delta T = -1$ °C		$\Delta T = \pm 0$ °C		$\Delta T = +1$ °C		$\Delta T = +2$ °C	
	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)
	0.00	48	0.00	49	0.00	50	0.00	51	0.00	52
	10.37	126	10.37	127	10.37	128	10.37	129	10.37	130
	12.00	178	12.00	179	12.00	180	12.00	181	12.00	182
	14.00	178	14.00	179	14.00	180	14.00	181	14.00	182
Critical resolution	1.06		1.10		1.13		1.09		1.15	

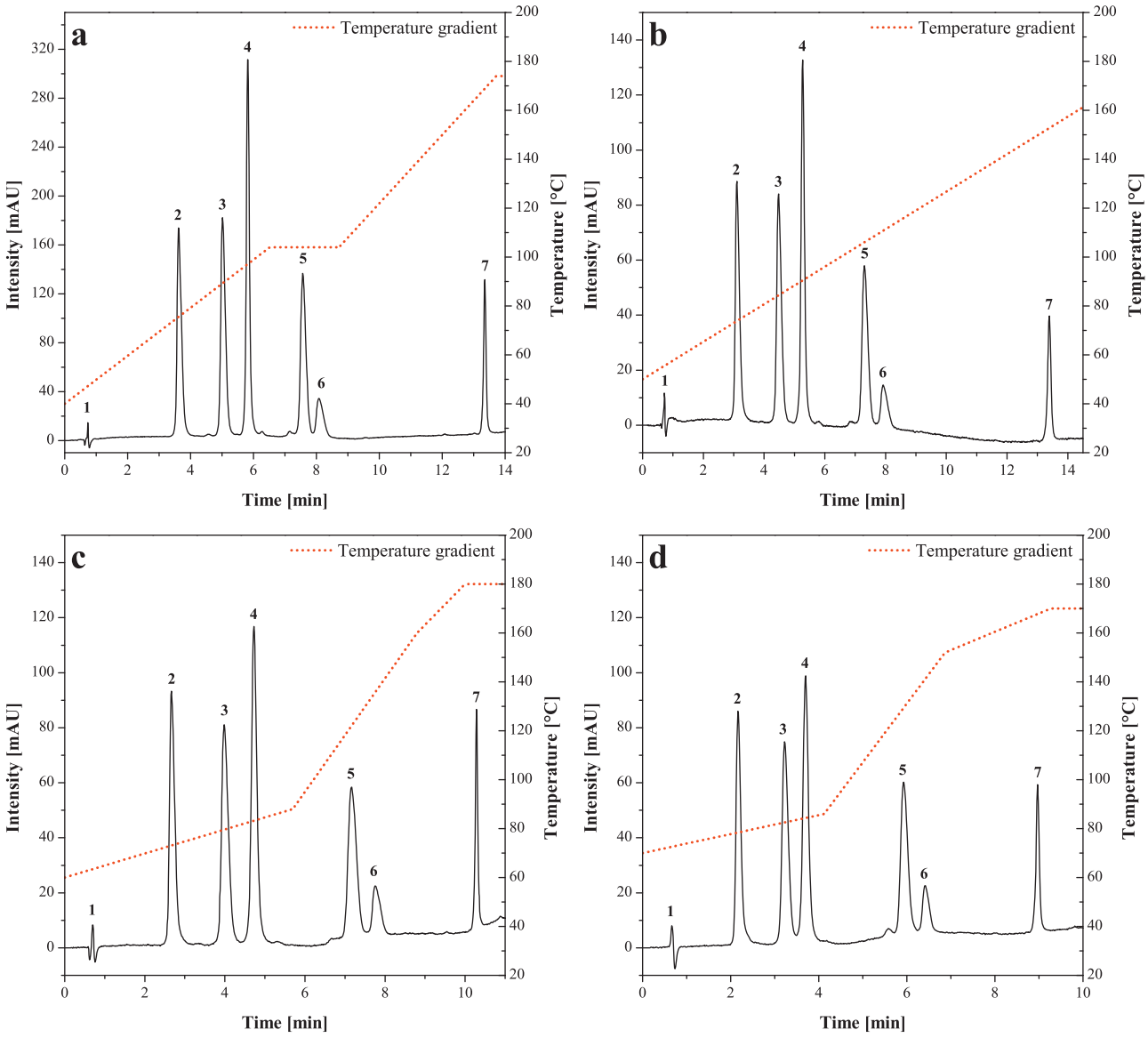

Fig. 3. Chromatograms of the separation of six food additives by temperature-gradient elution. Different start temperatures of the gradient were employed: (a) = 40 °C, (b) = 50 °C, (c) = 60 °C and (d) = 70 °C. Chromatographic conditions: Waters XBridge C-18 (50 mm × 3.0 mm, 3.5 μm); mobile phase: water + 0.1% formic acid; injection volume: (a) 2 μL, (b–d) 1 μL; temperature gradient: see figure. Analytes: (1) uracil, (2) theobromine, (3) theophylline, (4) catechine, (5) caffeine, (6) aspartame and (7) rutin.

4.5. Recommendations for temperature-programming method development

In order to assist the user to perform temperature-programming method development by means of the LES model, we are able to define the following recommendations.

First, perform two temperature-gradient runs at a low start temperature of, e.g., 40 °C as well as two gradient measurements at a higher start temperature of, e.g., 80 °C. If the user has information which might be a suitable temperature range for the start temperature of the resulting optimized temperature-gradient method, it would be advantageous when the temperature range between the upper and lower temperature would include the start temperature. In this case, values of S_T as well as k_0 would be calculated by means of an interpolation which should result in small errors of predicted retention times when compared to calculations by means of an extrapolation of these parameters. Moreover, it is also possible to choose start temperatures of e.g., 100 °C and 140 °C for the initial measurements, but it has to be considered that the useable temperature range would be restricted.

The slopes of the basic temperature-gradient measurements at different start temperatures should differ by a factor of at least three, for example, 2 °C min^{-1} and 6 °C min^{-1}. This recommendation is to be accounted for by the similarity of the linear elution strength (LES) and the linear solvent strength (LSS) relationship. The LSS theory assumes a linear relationship between the logarithm of the retention factor of a solute and the content of the organic solvent in the mobile phase [38]. In general, this is not precisely correct and curved plots will be observed [41–43]. In order to improve the accuracy of retention time predictions by means of the LSS model, it was recommended that the slopes of the measurements which have been employed for data fitting should differ by a factor of at least three [37,41]. In this context a similar issue exists in LES theory where a linear relationship between the logarithm of the retention factor of a solute and temperature is assumed. In the case of curved plots of ln k vs. T, the accuracy of retention time predictions might be improved when the slopes of the input temperature-gradient measurements differ by a factor of three.

Furthermore, it is important that the selected slopes of the temperature gradient measurements at the lower start temperature are

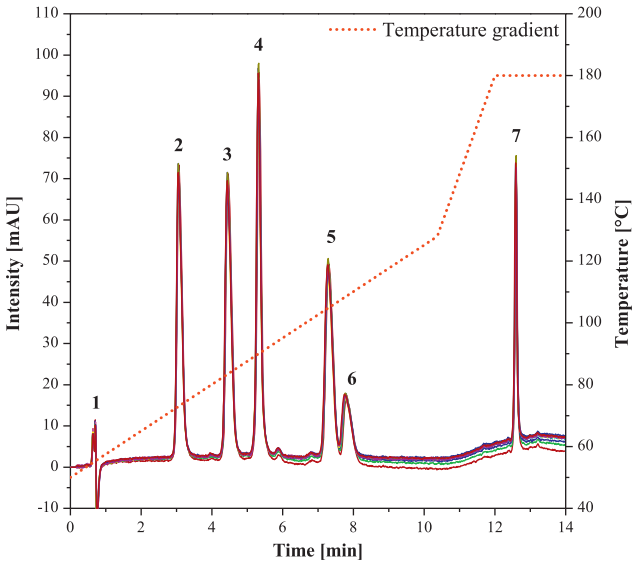

Fig. 4. Overlay of nine chromatograms of the separation of six food additives and uracil by temperature-gradient elution. Chromatographic conditions: Waters XBridge C-18 (50 mm × 3.0 mm, 3.5 μm); mobile phase: water + 0.1% formic acid; flow rate: 0.5 mL min^{-1}; injection volume: 1 μL; temperature gradient: 0 min at 50 °C; 10.37 min at 128 °C; 12.00 at 180 °C; 14.00 min at 180 °C. Analytes: (1) uracil, (2) theobromine, (3) theophylline, (4) catechine, (5) caffeine, (6) aspartame and (7) rutin.

equal to the slopes of the temperature gradients at the higher start temperature. Otherwise, the temperature dependent calculation of the LES parameters k_0 as well as S_T might fail.

Moreover, it is recommended that the analytes elute within the temperature-gradient window when performing the initial temperature-gradient measurements. For example, if a temperature gradient from 40 °C to 140 °C in 50 min (2 °C min^{-1}) is applied, the last eluting compound should be eluted from the column within 50 min. In the case, when an analyte elutes isothermally after the temperature-gradient, values of S_T and k_0 calculated as described in the theoretical section are less reliable. In other words, less reliable retention time predictions would be expected.

In order to summarize this section, the recommendations and the resulting experimental design are graphically represented in Fig. 5.

From a practical point of view, the first run should be performed at the lower start temperature with the higher gradient slope. It can be assumed that if the compounds elute within this gradient window, the analytes will also elute within the gradient window when

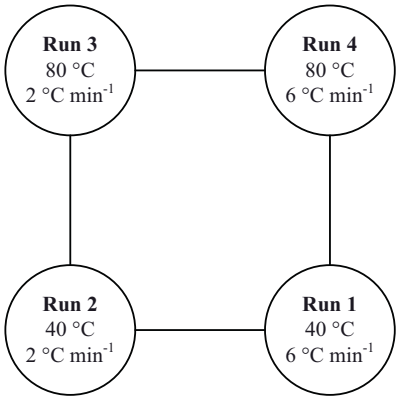

Fig. 5. Recommended experimental design to perform systematic temperature-programming method development by means of the LES model in high-temperature liquid chromatography.

the lower gradient slope or a higher start temperature is applied. In the case where the analytes do not elute within the gradient window, the user has to change the investigated temperature interval or steepness of the temperature gradients.

5. Conclusion

The results shown in this study clearly underline that retention time predictions by means of the LES model and four temperature-gradient input measurements are very suitable to perform systematic temperature-programming method development in high-temperature liquid chromatography. On the basis of the new experimental design, reliable retention time predictions with an average relative error less than 5% can be achieved.

Furthermore, the LES model in temperature-programmed LC works in isocratic operation mode. Hence, method development can also be performed in the case where an isocratic mobile phase consisting of water and an organic modifier is employed, which has been shown previously [28]. In addition, if the practitioner does not want to change the start temperature during method development, only two temperature-gradient input measurements are required to perform method development. Furthermore, the described temperature-programming approach is not only restricted to polar analytes such as sulfonamides [29] or food additives. The described methodology can also be applied to non-polar substances such as steroids [44] by using a column which is less hydrophobic than hybrid silica based C-18 columns. For this reason, metal oxide based columns such as polymer coated zirconium dioxide would be suitable.

Acknowledgments

The authors would like to thank for financial aid supported by the German Federal Ministry of Economics and Technology on basis of a decision by the German Bundestag. We would also like to thank Waters GmbH for the gift of the HPLC columns. Moreover, the authors would like to thank the reviewers for their valuable comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.018.

References

[1] C.L. Guillemin, J.L. Millet, J. Dubois, J. High Resolut. Chromatogr. Chromatogr. Commun. 4 (1981) 280.
[2] T. Teutenberg, P. Wagner, J. Gmehling, J. Chromatogr. A 1216 (2009) 6471.
[3] T. Teutenberg, S. Wiese, P. Wagner, J. Gmehling, J. Chromatogr. A 1216 (2009) 8470.
[4] T. Teutenberg, S. Wiese, P. Wagner, J. Gmehling, J. Chromatogr. A 1216 (2009) 8480.
[5] V. Heuer, M. Elvert, S. Tille, M. Krummen, X.P. Mollar, L.R. Hmelo, K.U. Hinrichs, Limnol. Oceanogr. Methods 4 (2006) 346.
[6] J.P. Godin, L.B. Fay, G. Hopfgartner, Mass Spectrom. Rev. 26 (2007) 751.
[7] J.P. Godin, G. Hopfgartner, L. Fay, Anal. Chem. 80 (2008) 7144.
[8] M. Roloff, H. Erfurt, G. Kindel, C.-O. Schmidt, G. Krammer, Process for the separation and sensory evaluation of flavours, in: World Intellectual Property Organization, WO 2006/111476 A1, 2006.
[9] K.V. Reichelt, R. Peter, S. Paetz, M. Roloff, J.P. Ley, G.E. Krammer, K.H. Engel, J. Agric. Food Chem. 58 (2010) 458.
[10] D. Louden, A. Handley, S. Taylor, I. Sinclair, E. Lenz, I.D. Wilson, Analyst 126 (2001) 1625.
[11] S. Saha, R.M. Smith, E. Lenz, I.D. Wilson, J. Chromatogr. A 991 (2003) 143.
[12] O. Chienthavorn, R.M. Smith, S. Saha, I.D. Wilson, B. Wright, S.D. Taylor, E.M. Lenz, J. Pharm. Biomed. Anal. 36 (2004) 477.
[13] X.Q. Yang, L.J. Ma, P.W. Carr, J. Chromatogr. A 1079 (2005) 213.
[14] D. Guillarme, S. Heinisch, J.Y. Gauvrit, P. Lanteri, J.L. Rocca, J. Chromatogr. A 1078 (2005) 22.
[15] R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, S.D. Taylor, Anal. Chem. 71 (1999) 4493.

[16] A.M. Edge, I.D. Wilson, S. Shillingford, Chromatographia 66 (2007) 831.
[17] I. Molnar, J. Chromatogr. A 965 (2002) 175.
[18] E.F. Hewitt, P. Lukulay, S. Galushko, J. Chromatogr. A 1107 (2006) 79.
[19] S. Heinisch, E. Lesellier, C. Podevin, J.L. Rocca, A. Tchapla, Chromatographia 44 (1997) 529.
[20] J.K. Törnblom, T.F.W. Bureyko, C.D. MacKinnon, J. Chromatogr. A 1095 (2005) 68.
[21] N.M. Djordjevic, F. Houdiere, P.F. Fowler, F. Natt, Anal. Chem. 70 (1998) 1921.
[22] F. Houdiere, P.W.J. Fowler, N.M. Djordjevic, Anal. Chem. 69 (1997) 2589.
[23] S.J. Marin, B.A. Jones, W.D. Felix, J. Clark, J. Chromatogr. A 1030 (2004) 255.
[24] S. Giegold, T. Teutenberg, J. Tuerk, T. Kiffmeyer, B. Wenclawiak, J. Sep. Sci. 31 (2008) 3497.
[25] P. Nikitas, A. Pappa-Louisi, K. Papachristos, C. Zisi, Anal. Chem. 80 (2008) 5508.
[26] A. Pappa-Louisi, P. Nikitas, C. Zisi, K. Papachristos, J. Sep. Sci. 31 (2008) 2953.
[27] J. Garcia-Lavandeira, P. Oliveri, J.A. Martinez-Pontevedra, M.H. Bollain, M. Forina, R. Cela, Anal. Bioanal. Chem. 399 (2011) 1951.
[28] S. Wiese, T. Teutenberg, T.C. Schmidt, Anal. Chem. 83 (2011) 2227.
[29] S. Wiese, T. Teutenberg, T.C. Schmidt, J. Chromatogr. A 1218 (2011) 6898.
[30] T. Teutenberg, K. Hollebekkers, S. Wiese, A. Boergers, J. Sep. Sci. 32 (2009) 1262.
[31] T. Teutenberg, H.J. Goetze, J. Tuerk, J. Ploeger, T.K. Kiffmeyer, K.G. Schmidt, W.G. Kohorst, T. Rohe, H.D. Jansen, H. Weber, J. Chromatogr. A 1114 (2006) 89.
[32] http://www.sim-gmbh.de/index.php?option=com_content&task=view&id=64&Itemid=502&lang=en, April 2011.
[33] D.E. Bautz, J.W. Dolan, W.D. Raddatz, L.R. Snyder, Anal. Chem. 62 (1990) 1560.
[34] D.E. Bautz, J.W. Dolan, L.R. Snyder, J. Chromatogr. 541 (1991) 1.
[35] A. Jayatilaka, C.F. Poole, J. Chromatogr.: Biomed. Appl. 617 (1993) 19.
[36] J.P. Larmann, J.J. Destefano, A.P. Goldberg, R.W. Stout, L.R. Snyder, M.A. Stadalius, J. Chromatogr. 255 (1983) 163.
[37] M.A. Quarry, R.L. Grob, L.R. Snyder, Anal. Chem. 58 (1986) 907.
[38] L.R. Snyder, J.W. Dolan, High-Performance Gradient Elution—The Practical Application of the Linear-Solvent-Strength Model, Wiley-Interscience, John Wiley & Sons, Inc., Hoboken, NJ, 2007.
[39] L.R. Snyder, in: C. Horvath (Ed.), High-Performance Liquid Chromatography, Advances and Perspectives, Academic Press, New York, 1980, p. 207.
[40] L.R. Snyder, J.W. Dolan, Adv. Chromatogr. 38 (1998) 115.
[41] J.W. Dolan, D.C. Lommen, L.R. Snyder, J. Chromatogr. 485 (1989) 91.
[42] P.J. Schoenmakers, H.A.H. Billiet, R. Tijssen, L. Degalan, J. Chromatogr. 149 (1978) 519.
[43] P.J. Schoenmakers, H.A.H. Billiet, L. Degalan, J. Chromatogr. 185 (1979) 179.
[44] T. Teutenberg, High-Temperature Liquid Chromatography—A User's Guide for Method Development, Royal Society of Chemistry, Cambridge, 2010.