A General Strategy for Performing Temperature Programming in High Performance Liquid Chromatography

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„Es kommt nicht darauf an,

mit dem Kopf durch die Wand zu rennen,

sondern mit den Augen die Tür zu finden.“

Werner von Siemens

Abstract

The use of elevated temperature or temperature programming in liquid chromatography provides several advantages such as fast analysis, increased efficiency, a change of selectivity and an increase of the elution strength of the mobile phase. Method development in high-temperature liquid chromatography is usually governed by trial and error although a systematic approach is preferred. Therefore, it was investigated whether the empirical linear elution strength (LES) retention model can be adapted from temperature-programmed gas chromatography (GC) to temperature-programmed liquid chromatography (LC). It was found that by means of the LES model, retention times of selected steroids and polycyclic aromatic hydrocarbons can be precisely predicted depending on a simple linear temperature gradient in LC. An average relative error of less than 2% of predicted retention times was observed. Moreover, the influences of column chemistry, inner column diameter and composition of an isocratic mobile phase were studied. Because of these findings, the LES model was further extended in order to predict more complex segmented temperature gradients. For these gradients, the retention times of sulfonamides could be predicted precisely with an average relative error of 2.2%. The LES model in GC permits isothermal retention time predictions on the basis of temperature-gradient measurements. This approach was also employed in liquid chromatography and it is shown that this assumption cannot be transferred to temperature-programmed LC. Because of the need to predict isothermal retention times, predictions based on a plot of the natural logarithm of the retention factor were tested for temperature dependency. It was found that a plot of the natural logarithm of the retention factor versus temperature yields reliable isothermal retention time predictions. In order to improve the accuracy of retention time predictions based on temperature gradients even further, a second compound specific model parameter was also calculated temperature dependent. Using this approach, the relative error of retention time predictions of multi-step temperature gradients can be decreased to around 1.5%. Concurrently, a new experimental design was introduced which permits isothermal predictions on the basis of only four temperature-gradient input measurements. Moreover, a set of recommendations to assist the practitioner during method development in HT-HPLC was established. Finally, the linear solvent strength and the linear elution strength retention model were combined in order to predict simultaneous solvent and temperature gradients in LC. An average relative error of 0.6% of predicted retention times was observed. On the basis of the present work, temperature gradients can now be incorporated in systematic method development in liquid chromatography.

Kurzfassung

In der Flüssigchromatographie (LC) bietet die Anwendung höherer Temperaturen oder der Temperaturprogrammierung verschiedene Vorteile. Trennungen können beschleunigt, die Effizienz kann erhöht und die Selektivität sowie die Elutionsstärke der mobilen Phase kann beeinflusst werden. Dennoch wird die Methodenentwicklung in der Hochtemperatur-LC nicht systematisch durchgeführt. Im Rahmen dieser Arbeit ist untersucht worden, ob das empirische *linear elution strength* (LES) Retentionsmodell aus der temperaturprogrammierten Gaschromatographie (GC) auf die temperaturprogrammierte LC übertragen werden kann. Dazu wurde das LES Modell verwendet, um Retentionszeiten von ausgewählten Steroiden und polyzyklischen aromatischen Kohlenwasserstoffen in Abhängigkeit eines Temperaturgradienten zu simulieren. Die Retentionszeiten der Analyten konnten mit einem mittleren relativen Fehler von weniger als 2% präzise vorhergesagt werden. Gleichzeitig wurden die Einflüsse der Säulenchemie, des Säuleninnendurchmessers und die Zusammensetzung isokratischer mobiler Phasen untersucht. Durch die anschließende Erweiterung des LES Modells konnten auch komplexe mehrstufige Temperaturgradienten präzise simuliert werden. Die Retentionszeiten von Sulfonamiden konnten mit einem mittleren relativen Fehler von 2,2% vorhergesagt werden. In der GC kann das LES Modell auch zur Simulation von isothermen Trennungen auf Basis von Temperaturgradienten verwendet werden. Dieser Ansatz konnte jedoch nicht auf die LC übertragen werden. Da die Simulation von isothermen Retentionszeiten erforderlich ist, wurden verschiedene Auftragungen des Logarithmus des Retentionsfaktors in Abhängigkeit von der Temperatur untersucht. Die Auftragung des Logarithmus des Retentionsfaktors gegen die Temperatur führt zu vertrauenswürdigen Vorhersagen. Um die Genauigkeit der Simulationen weiter zu verbessern, wurde ein zusätzlicher analytabhängiger Modellparameter temperaturabhängig berechnet. Dadurch konnte der relative Fehler der Vorhersage von mehrstufigen Temperaturgradienten um 1,5% gesenkt werden. Gleichzeitig wurde eine neue Kombination von Basismessungen vorgestellt mit der es möglich ist, isotherme Trennungen auf Basis von vier Temperaturgradienten vorherzusagen. Weiterhin wurden Empfehlungen formuliert, um den Anwender während der Methodenentwicklung in der Hochtemperatur-LC zu unterstützen. Abschließend wurden das *linear solvent strength* und das *linear elution strength* Retentionsmodell kombiniert, um simultane Lösungsmittel- und Temperaturgradienten zu simulieren. Der mittlere relative Fehler dieser Vorhersagen betrug 0,6%. Auf Grundlage dieser Arbeit ist es nun möglich, Temperaturgradienten als Parameter einer systematischen Methodenentwicklung in der Hochtemperatur-Flüssigchromatographie zu berücksichtigen.

Contents

Chapter 1. General Introduction

The use of elevated temperature and temperature programming in high performance liquid chromatography (HPLC) provides several advantages [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]. Temperature is usually discussed in terms of speeding up a separation [5-7], because elevated temperature yields a lower viscosity of the mobile phase, which concurrently results in a lower system backpressure and a higher diffusivity of the analytes. Thus, the flow rate can be increased in order to decrease the total analysis time. Moreover, an increase of temperature yields also a flattened van Deemter curve. Thus, the increased flow rate only slightly affects the column efficiency [8,9]. Furthermore, the reduced backpressure enables the use of smaller particles or longer columns [10].

Temperature, however, is not only a tool to speed up a separation or to increase efficiency; temperature also plays an important role in changing selectivity in liquid chromatography, especially for polar and ionizable compounds [11,12]. Chen and Horvath compared solvent gradient elution and temperature programming for the separation of selected alkylbenzenes and proteins [13]. They concluded that temperature programming can only be an insufficient alternative to solvent gradient elution. However, they also pointed out that temperature programming in combination with solvent gradient elution could be employed as fine tuning to enhance the critical resolution of structurally similar macromolecules such as proteins. Another impressive example was given by Vanhoenacker et al. [8] who developed a method for the separation of 20 pesticides using a combined solvent and temperature gradient. Vanhoenacker was able to show that an isothermal baseline separation within a temperature interval from 40 °C to 90 °C by means of a solvent gradient was not possible, and a combination of simultaneous solvent and temperature programming was required for a baseline separation. A similar example was given by Giegold et al. [14] who could also show that a baseline separation of eight sulfonamides and trimethoprim was only possible in dual gradient mode, where a solvent and temperature gradient were applied simultaneously.

Another important temperature depending change in the properties of water and binary hydro organic solvent mixtures is related to the static permittivity that decreases with increasing temperature [4]. In other words, the higher the temperature of the mobile phase, the lower is its polarity. Therefore, under certain conditions, temperature gradients can be employed

instead of solvent gradients, as has been shown previously [15-19]. The fact that the elution strength of the mobile phase can be tuned by temperature during a chromatographic run enables new special hyphenation techniques [18-23]. Most of these hyphenation techniques require the use of a water mobile phase or a minimized content of organic solvent in the mobile phase. Three of these techniques will be described in more detail to demonstrate why temperature gradients in LC are required.

The first high-temperature high performance liquid chromatography (HT-HPLC) hyphenation technique which will be discussed in the present work has been presented by de Boer and Irth [20] and deals with the online determination of biologically active compounds in complex mixtures. This was achieved by the hyphenation of HT-HPLC with a continuous-flow enzyme-substrate reaction where the reaction products are detected by electrospray-ionization mass spectrometry (ESI-MS). The determination of the biological activity is based on an indirect approach because the inhibition of the enzyme activity is measured by compounds eluting from the HPLC column. This yields a temporary change in product concentration and is recorded as a negative peak by ESI-MS. The main parts of the system are two reaction coils. In the first one the eluting components from the HPLC column are mixed with the enzyme and the enzyme activity might be inhibited if the analytes are biologically active. Afterwards, the substrate is added and a reaction between enzyme and substrate takes place in the second reaction coil yielding two products. If an inhibition of the enzyme activity by the analytes takes place, a decrease of the measured concentrations of these two products will be observed. Because of the online concept there is an inherent limitation which has to be overcome. The concentration of the organic solvent in the mobile phase should not exceed a certain concentration; otherwise the enzyme activity would be affected. In order to decrease the necessary organic content in the mobile phase, high-temperature HPLC was employed to elute also non-polar analytes.

Another interesting technique where temperature plays an important role is the so-called LC taste which has been patented by Symrise [21,22]. This technique also employs high-temperature HPLC for the determination of gustatory active compounds in complex mixtures. For better understanding the system setup will be described briefly. The HPLC system consists of two pumps to enable solvent gradient programming and a high-temperature column oven with eluent cooling unit which is used to cool down the mobile phase after the separation. Afterwards, the effluent is directed to a nondestructive detector such as an ultraviolet (UV), a diode array (DAD) or a refractive index (RF) detector. After passing one

of these detectors a flow splitter is employed in order to split the mobile phase into two pathways. The eluate can then be directly tasted by a person and detected with destructive detection techniques such as mass spectrometry (MS) or evaporative light-scattering (ELS). Usually, flavors are complex mixtures of compounds with different polarity. This means the polarity of the mobile phase has to be changed during the chromatographic run to separate these analytes. This can be easily performed by solvent gradients with an organic modifier such as acetonitrile, methanol or tetrahydrofuran. However, these solvents cannot be employed when a direct online tasting is carried out by a human being. Hence, water will be the preferred solvent, because it is not toxic and can also be used with the other detection techniques described above. Unfortunately, for some non-polar compounds the elution strength of water at elevated temperatures is not sufficient to elute these from the column. Therefore, solvent gradient elution is inevitable to increase the elution strength of the mobile phase. In this case, ethanol is a suitable organic solvent which significantly enhances the elution strength in solvent gradient mode. However, ethanol can only be used up to a certain concentration of 5% to 30%. Otherwise, it has a negative impact on sensory impression. Therefore, a combination of solvent and temperature programming is necessary to achieve the desired separation where the ethanol content in the mobile phase will not falsify the sensory impressions.

The last technique which should be mentioned is the hyphenation of HPLC with isotope ratio mass spectrometry (IRMS). With this technique, the abundance ratio of stable isotopes of elements is determined. For example, the ratio of carbon isotopes 13 to 12 ($^{13}C/^{12}C$) is detected. Carbon isotope analysis by hyphenated HPLC-IRMS can be performed using the LC-IsoLink by Thermo [23]. For a better understanding this hyphenation will be described in more detail. First, the HPLC-separated compounds are introduced within the mobile phase into a heated zone. Here, wet oxidation of the entire organic carbon takes place to yield carbon dioxide at approximately 100 °C. The CO_2, which is dissolved in the mobile phase, is then directed to a separation unit where the carbon dioxide is separated via a membrane from the mobile phase. Afterwards, the CO_2 is dried in a gas dryer and then directed to the isotope ratio mass spectrometer where the abundance ratio of $^{13}C/^{12}C$ is determined. Because of the experimental setup, this hyphenation has some restrictions which have to be considered. The most important point is that the mobile phase has to be absolutely free of any carbon or carbon containing compounds such as organic buffers, because these compounds are also fully converted to carbon dioxide and will falsify the measured isotopic composition of the

analytes. Also, in LC-IRMS analytes with different polarity have to be separated. Therefore, the polarity of the mobile phase has to be varied in order to elute all analytes from the column. In conventional HPLC organic solvent gradients are employed to alter the elution strength of the mobile phase during a chromatographic run. However, in the case of LC-IRMS this cannot be accomplished. The optimization of the chromatographic separation can only be performed by temperature and organic solvent gradients have to be completely replaced by temperature gradients. Furthermore, LC-IRMS requires a baseline separation. Because of the isotope effects observed in chromatography the measured isotope composition of analytes in partially resolved peaks will otherwise be falsified [24].

The three hyphenation techniques described above clearly underline that temperature programming is absolutely mandatory to achieve a suitable separation. This requirement poses a further problem. The practitioner has to develop a separation method where a temperature gradient is employed instead of a solvent gradient. Several examples are given in literature of method development based on temperature programming in liquid chromatography [8,13,14,25-29]. However, it has to be considered that method development during these studies was governed by trial and error. This is a general issue during method development in liquid chromatography and not restricted to temperature programming. Usually, the user changes different chromatographic parameters such as solvent gradient, pH or temperature according to his or her experience with the aim to increase the chromatographic resolution while simultaneously decreasing the analysis time. This approach needs a lot of time and financial resources. To overcome these deficits, a structured method development is mandatory. Several modeling software packages, such as ChromSwordAuto [30], DryLab [31], Osiris [32] or ACD/LC & GC Simulator [33] are commercially available to reduce the necessary experiments and to assist the user. On the basis of the employed retention models of these software packages, it is possible to predict the retention of the analytes with high accuracy. Solvent gradient steepness (% B) and pH of the mobile phase or solvent gradient steepness (% B) and temperature (T) are often optimized simultaneously [34,35]. The software packages mentioned before do not permit retention time predictions depending on a temperature gradient in LC. This problem was first considered by Nikitas and Pappa-Louisi. They developed prediction models which permit simulation of retention times when solvent composition and temperature are changed simultaneously [36,37]. Up to now, their models were tested using only linear temperature gradients within a relatively small temperature interval from 15 °C to 75 °C where moderate gradient slopes from 2 °C min^{-1} up

to 10 °C min^{-1} were applied. Furthermore, no software package is commercially available which has implemented these retention models. Recently, Cela and co-workers have described computer-assisted method development in high-temperature liquid chromatography based on an evolutionary algorithm [38]. The developed approach also permits dual mode simulations of retention times when solvent composition and temperature are changed simultaneously. In 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 [38]. However, based on the data given by Cela and co-workers a relative error up to 10% was calculated for simultaneous solvent and temperature-gradient predictions.

Working at elevated temperature in liquid chromatography requires that the mobile phase is adequately preheated before it enters the column inlet. Otherwise, peaks will be severely distorted or even split [39]. To overcome this problem, some manufacturers have commercialized specially designed high-temperature column ovens such as the SIM HT-HPLC 200 column oven [40] or the Polaratherm oven [41]. Both heating systems are depicted in Figure 1-1.

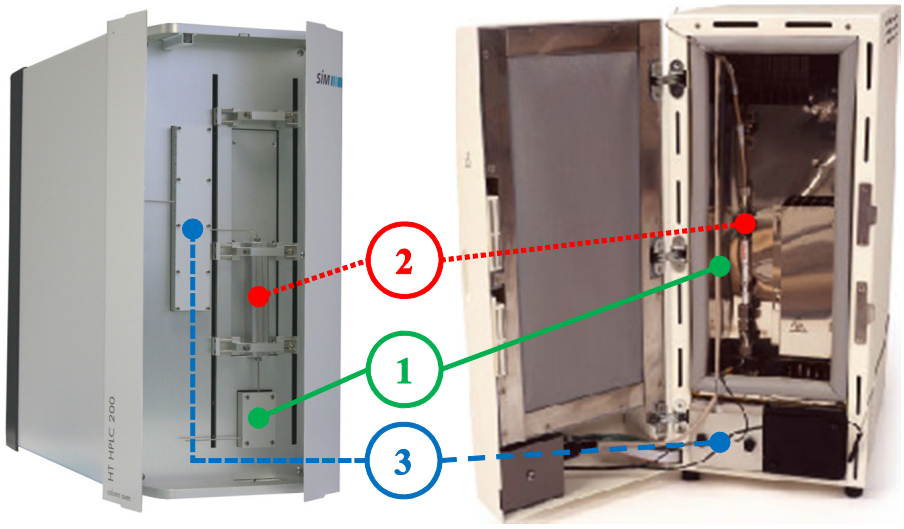

Figure 1-1: Left hand side: SIM HT-HPLC 200 column oven by SIM (SIM-Scientific Instruments Manufacturer, Oberhausen, Germany); picture copyright SIM. Right hand side: Polaratherm 9000 by Selerity Technologies, Inc., (Salt Lake City, USA); picture copyright Selerity Technologies. 1: eluent preheating unit, 2: column heating unit, 3: eluent cooling unit.

On the left hand side the SIM HT-HPLC 200 column oven (Figure 1-1) is displayed. Here, the heat transfer is obtained by block heating which means that the capillaries and the column are tightly enclosed by aluminum blocks. This modular system consists of the eluent preheating

unit (1), the column heating unit (2), and the eluent cooling unit (3). In contrast, on the right hand side of Figure 1-1 the Polaratherm high-temperature column oven is shown. Here, the heat transfer is achieved by forced-air convection (air-bath), the same concept employed in temperature-programmed GC. This system consists also of a preheating unit (1), a convection oven where the column is placed (2), and an eluent cooling unit (3).

The main difference between both column ovens is the approach to heat the mobile and stationary phase which provides some advantages as well as drawbacks. The advantages of column ovens based on the forced-air convection concept is that these heating systems are rather simple, cheap, and the column can be installed very easy [1]. However, the main drawback of these thermostats is the poor heat transfer between the heated air and the metal surface of the column. The result will be thermal lags between the programmed and the actual temperature in the oven as well as between the actual temperature inside the oven and the effective temperature in the middle of the column packing [36]. These thermal lag phenomena will also be observed when the oven and the column are cooled down after a temperature gradient which results in long re-equilibration times [1]. In contrast, if a column oven based on block heating is employed, thermal lag phenomena are less pronounced when compared to air-bath heating systems. Here, the column and the capillaries are tightly enclosed by aluminum blocks. Hence, the heat transfer between the column and the heating unit is most efficient [1]. In other words, the heat at the outer side of the column is transferred quickly to the middle of the column packing so that the programmed temperature gradient closely matches the gradient that the analyte experiences in the column. However, due to the wide range of column dimensions and designs of the manufacturers, the column shells have to be tailor made for a different column manufacturer which is a drawback of column ovens based on block heating [1]. Moreover, the change of the column is more difficult when compared to forced-air convection column ovens.

Another prerequisite of the use of elevated temperature in LC is a temperature stable column. Conventional silica-based C_{18} HPLC columns are not stable at high temperatures which results in an immediate decrease of column performance [42,43]. To overcome the limitations of silica-based stationary phases, metal oxide based columns have been developed. Packing material based on zirconium and titan oxide exhibit a high mechanical, temperature, and pH stability. Furthermore, these oxides can be coated with polymers such as polybutadiene (PBD) and then employed for reversed phase separations [42,43]. Therefore, columns of this type were used for separations at temperatures as high as 200 °C [44-46]. However, with the

introduction of HPLC systems with an enlarged pressure range, column packings which are stable at pressures up to 1,000 bars were required. Therefore, some column manufacturers have introduced silica-based hybrid packings which are stable at high inlet pressures. Because of the chemical modification to obtain high pressure stability, the temperature and the pH stability was also significantly increased. Up to now, several silica-based columns are commercially available which can be used at high temperatures [47-53]. In other words, there is no reason why high-temperature liquid chromatography should not be implemented in routine laboratory practice, because all required hardware parts such as a high-temperature column oven or temperature stable columns are commercially available.

Scope of the Study

This study is focused on the development of an approach which permits temperature-programming method development in high-temperature liquid chromatography.

In Chapter 2, it is investigated whether the linear elution strength (LES) retention model can be adapted from temperature-programmed gas chromatography to temperature-programmed liquid chromatography. The application of the LES model in high-temperature liquid chromatography has been discussed in terms of the influence of the column chemistry, the column inner diameter, the composition of the mobile phase, and the temperature gradient-steepness using two mixtures consisting of steroids and polycyclic aromatic hydrocarbons (PAHs).

Chapter 3 addresses the extension of the adapted linear elution strength (LES) model in order to predict more complex segmented temperature gradients in LC. Here, selected sulfonamides are used as model analytes. Furthermore, the ability to predict isothermal separations based on temperature-gradient input runs as well as on isothermal measurements is investigated. Both approaches are discussed in terms of the accuracy of predicted retention times and practical considerations. Moreover, the systematic method development for the separation of five sulfonamides by temperature-programmed LC is performed based on the current stage of development of the LES retention model.

Chapter 4 describes how the accuracy of retention time predictions based on the linear elution strength retention model can be further increased if another solute constant is calculated temperature dependent. Furthermore, an approach is discussed which permits predictions of

isothermal retention times based on temperature-gradient measurements. Moreover, the accuracy of retention time predictions and the practical application of the new experimental design are investigated by method development for analysis of selected food additives. Finally, a set of recommendations to assist the practitioner during systematic method development in HT-HPLC is established.

Retention time predictions based on temperature gradients in liquid chromatography described in Chapters 2 to 4 are performed under isocratic condition. Chapter 5 deals with the combination of the linear solvent strength (LSS) and the linear elution strength (LES) model in order to predict simultaneous solvent and temperature gradients in liquid chromatography. In addition, the described approach for these predictions is discussed in terms of the accuracy of dual mode retention time predictions, practical application, and compared with retention models found in literature.

In Chapter 6, the findings of this work are summarized and an outlook on further investigations is given.

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Chapter 2. A General Strategy for Performing Temperature Programming in High Performance Liquid Chromatography – Prediction of Linear Temperature Gradients*

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2.1 Introduction

It is well documented that increasing the temperature will result in a change of the physicochemical properties of water and binary solvent mixtures [1-3]. Temperature is usually discussed in terms of speeding up a separation, because elevated temperature yields a lower viscosity of the mobile phase which concurrently results in a higher diffusivity of the analytes. Hence, the flow rate can and should be increased in order to operate the column in its respective van Deemter minimum [4-6]. Another change in the properties of water and binary hydro organic solvent mixtures is related to the static permittivity that decreases with increasing temperature [3]. In other words, the higher the temperature of the mobile phase, the lower is its polarity. Therefore, under certain conditions, temperature gradients can be employed instead of solvent gradients as has been shown previously [7-11].

The fact that the elution strength of a water mobile phase can be tuned by temperature during a chromatographic run enables new special hyphenation techniques. Most of these require the use of a water mobile phase or a minimized content of organic solvent in the mobile phase [12-16]. In these papers, method development is based on temperature programming because water is the preferred solvent. In order to obtain a good separation, method development is carried out by trial and error approaches. The user changes different chromatographic parameters such as solvent gradient, pH, or temperature according to his or her experience with the aim to increase the chromatographic resolution while simultaneously decreasing the analysis time. This approach needs a lot of time and financial resources. To overcome these deficits, a structured method development is mandatory. Several modeling software packages, e.g., DryLab [17], ChromSwordAuto [18], Osiris [19] or ACD/LC & GC Simulator [20], are commercially available to reduce the necessary experiments and to assist the user. On the basis of the employed retention models of these software packages, it is possible to predict the retention of the analytes with high accuracy. Solvent gradient steepness (% B) and pH of the mobile phase or solvent gradient steepness (% B) and temperature (T) are often optimized simultaneously [21,22]. To the best of our knowledge, however, there is no commercially available software package that predicts the retention of the analytes depending on a temperature gradient in LC. In order to facilitate the use and broaden the acceptance of these special hyphenation techniques, which are already implemented in industry, a systematic approach for temperature programming in liquid chromatography to assist the practitioner is necessary [14,23]. Previously, Nikitas and Pappa-Louisi [24,25] developed retention models which permitted retention time predictions for simultaneous solvent and temperature

gradients. However, their models were only tested using temperature gradients with moderate slopes from 2 °C min^{-1} up to 10 °C min^{-1} in a small temperature range from 15 °C up to 75 °C. Therefore, the aim of the present work was to investigate whether the linear elution strength (LES) approximation from temperature-programmed GC can also be employed in temperature-programmed liquid chromatography to allow for the prediction of retention times over a broad temperature range from 50 °C up to 180 °C for high-temperature LC and using steeper temperature gradients than reported so far. In addition, the validity of the model should be evaluated under different chromatographic conditions. The influences of the column chemistry, the column inner diameter, the composition of the mobile phase, and the gradient steepness were studied using test mixtures consisting of steroids and polycyclic aromatic hydrocarbons (PAHs).

2.2 Theoretical Basis

In this work, the linear elution strength (LES) retention model [26-29] was employed, which is similar to the widely used linear solvent strength model (LSS) [30,31] in solvent gradient liquid chromatography. The LES model was introduced in 1990 by Snyder and co-workers and is used for prediction of retention times of analytes in temperature-programmed gas chromatography (GC). Moreover, the LES model was implemented in the commercially available software DryLab 2000 plus for structured method development in GC. The prediction of solute retention depending on experimental temperature gradients can be described as follows [26]:

$$t_R = \frac{t_0}{b_T \ln(10)} \ln\left[e^{\ln(10) b_T} \left(k_0 + 1\right) - k_0 \right] \tag{2-1}$$

$$\text{with } b_T = \frac{t_0 S_T \Delta T}{T_G} \tag{2-2}$$

where t_R is the retention time of the solute, t_0 is the column dead time and k_0 is the retention factor of the solute corresponding to the start temperature of the temperature gradient. The temperature programming-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 T_G. Analogous to the LSS model, only two initial gradient runs have to be made. There are some recommendations to perform these measurements [26]. First, the gradient slopes of the input runs should differ by a factor of three, keeping all other experimental conditions constant.

Second, for reliable predictions, the analytes should elute in the temperature-gradient window [32]. In other words, if an input run is performed from 40 °C up to 160 °C in 20 min, the analytes should elute within 20 min. On the basis of these two initial runs, values of b_T or rather S_T and k_0 for each analyte are derived by a numerical solution of equations 2-1 and 2-2 using the Microsoft Excel solver. This approach is very similar to numerical solutions of the LSS relationship [33-35].

It is generally recognized that working at elevated temperatures in LC requires that the mobile phase is adequately preheated before it enters the column. Otherwise, peaks will be severely distorted or even split [36]. Another problem can arise if the column is operated at extremely high pressures of about 1000 bar. In this case, frictional heating can occur which also might yield band broadening, depending on the type of column oven [37-39]. In the case of temperature programming in liquid chromatography, another important aspect concerning the column oven has to be considered. In liquid chromatography, usually air-bath column ovens based on a similar concept as GC column ovens are used. In this case, the column is placed in the oven and the temperature of the ambient air is controlled. If temperature gradients are applied, radial temperature gradients will occur, which are called hysteresis phenomena [24] or thermal lag. At the start of the temperature gradient, the column wall is heated up by the surrounding medium which can be air, a water bath or a heated metal block. After a certain time is elapsed, the heat is transferred from the column wall to the stationary phase within the column. An efficient heat transfer can be obtained for a heated metal block which tightly encloses the column. In contrast, a much slower heat transfer is observed if air is used as the heating medium. The result of the thermal lag is schematically shown in Figure 2-1 by a comparison of the programmed and the effective temperature gradient in the center of the packing.

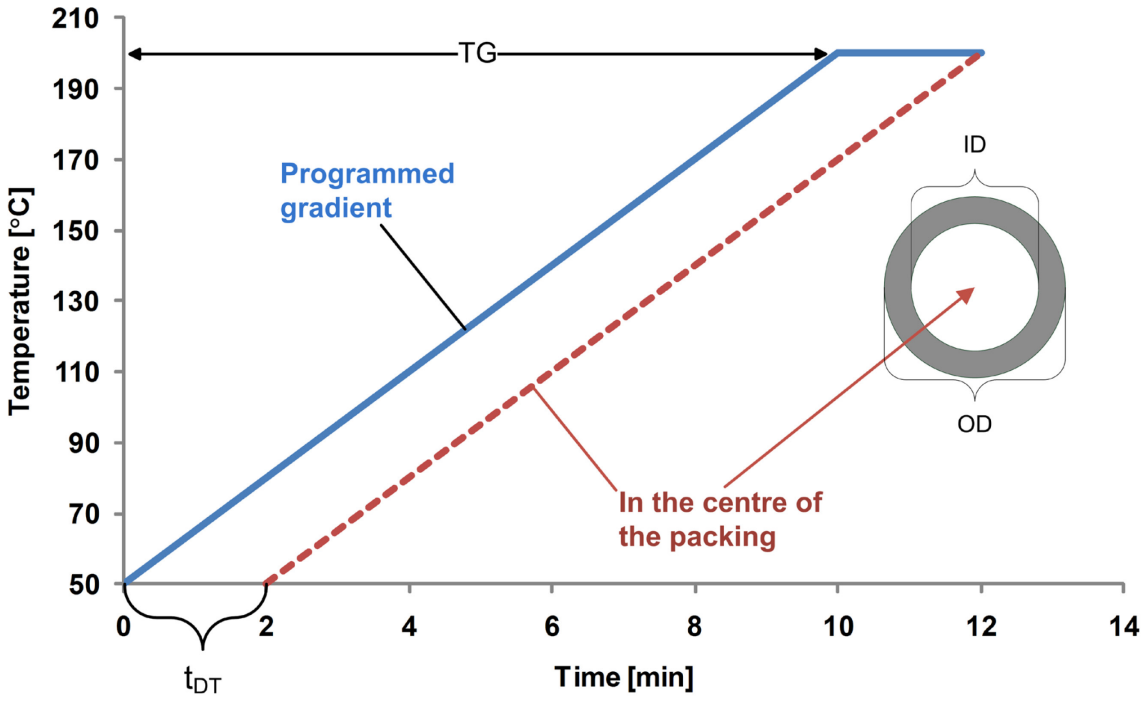

Figure 2-1: Schematic illustration of a temperature-dependent delay time (t_{DT}); TG: temperature-gradient time; ID: inner column diameter; OD: outer column diameter.

The best way to determine the thermal lag between the programmed and effective temperature gradient inside the column would be to measure the effective temperature in the center of the column packing. From a practical point of view, however, this is very difficult to achieve in a high pressure system with temperatures up to 200 °C. Because of this practical limitation, this paper is focused on the simulation of temperature gradients according to the LES model and a comparison between predicted and experimental retention times under temperature-gradient conditions. Moreover, it will be investigated how the temperature-dependent delay between the programmed and the effective temperature gradient can be taken into account using the LES relationship.

2.3 Experimental Section

2.3.1 Chemicals

High-purity deionized water was prepared by an Elix 10-Milli-Q Plus water purification system (Millipore, Eschborn, Germany). Acetonitrile (Optigrade) as well as methanol (Optigrade) were purchased from LGC Standards (Wesel, Germany). In this work, two different analyte mixtures were used. The first one was a mixture of four steroids which included 19-nortestosterone, testosterone, trans-dehydroandrosterone, and epitestosterone. The second one was a mixture of six polycyclic aromatic hydrocarbons which consisted of naphthalene, acenaphthylene, fluorene, anthracene, pyrene, and chrysene. All chemicals employed in this study except for the solvents were purchased from Sigma-Aldrich (Seelze, Germany) and of p. a. grade.

2.3.2 HPLC System

Two different HPLC systems (Beckman Gold and Shimadzu LC 10) were used to collect the chromatographic data. The Beckman System Gold HPLC (Beckman, Krefeld, Germany) consisted of a System Gold 126 pump, an AS 502e autosampler, and a System Gold 168 diode array detector. Additionally, a PL-ELS 1000 evaporative light-scattering detector (ELSD) from Polymer Laboratories (Polymer Laboratories Ltd., Darmstadt, Germany) was used and connected to the HPLC system via an SS420x AD Box (Scientific Instruments, Inc., West Palm Beach, USA). The Shimadzu LC 10 (Shimadzu, Duisburg, Germany) consisted of two LC-10AD$_{VP}$ pumps, a DGU-14 A degasser, an SIL-10AD$_{VP}$ autosampler, an SPD-M10A$_{VP}$ diode array detector, and an SCL-10A$_{VP}$ controller. A 500 psi back pressure regulator (GammaAnalysenTechnik, Bremerhaven, Germany) was connected behind the UV detector to keep the mobile phase in the liquid state. For data acquisition and analysis, Shimadzu LC solution (version 1.21 SP 1) and Beckman 32 Karat (version 7.0 Build 1048) were used.

2.3.3 Heating System

To heat the mobile and stationary phase, a commercially available SIM HT-HPLC 200 high-temperature column oven was used (SIM, Scientific Instruments Manufacturer, Oberhausen, Germany) [40,41]. The heating system was specially 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 the column are tightly enclosed by aluminum 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. In order to compensate effects which are related to frictional heating, the practitioner can define a temperature difference between the eluent preheating and the column heating unit. 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 of the preheating unit and the column were identical.

2.3.4 Temperature-Gradient Measurements

Steroid Mixture

For the steroid mixture, a temperature range from 60 °C up to 160 °C was investigated. In this range, four temperature gradients with slopes of 1.5 °C min^{-1}, 3.0 °C min^{-1}, 4.0 °C min^{-1}, and 6.0 °C min^{-1} were set using a water mobile phase and two ZirChrom-PDB columns (150 × 3.0 mm, 5 µm and 100 × 1.0 mm, 3 µm). The flow rate was set to 0.1 mL min^{-1} and 1.0 mL min^{-1} for the 1 mm ID and 3 mm ID column, respectively, and a volume of 20 µL was injected. UV detection was performed at 200 nm and 254 nm. In addition, the ELS detector was used because of the poor UV absorption of selected steroids.

PAH Mixture

For the PAH mixture, the temperature range was set from 50 °C up to 180 °C. Within this temperature range, gradients with slopes of 5 °C min^{-1} up to 30 °C min^{-1} with an interval of 2.5 °C min^{-1} were applied for each column. Here, a Waters Acquity Phenyl (100 × 2.1 mm, 1.7 µm) and a Waters XBridge C_{18} (75 × 4.6 mm, 2.5 µm) column were chosen as stationary phases. In this case, different mobile phases containing an organic modifier were used. For the measurements on the Waters Acquity Phenyl column, a mobile phase consisting of 50/50 (v/v) water/acetonitrile was employed at a flow rate of 0.3 mL min^{-1}. A mixture of 30/70 (v/v) water/methanol was used for the measurements with the Waters XBridge C_{18} column at a flow rate of 1.0 mL min^{-1}. UV detection of selected PAHs was carried out at a wavelength of 254 nm, and the injection volume was set to 1 µL for the 2.1 mm ID column and 3 µL for the 4.6 mm ID column.

2.4 Results and Discussion

The prediction of temperature gradients using the LES model does not include a temperature-dependent delay time. If a delay time exists, the LES model would not be able to match the experimental retention times. This also means the differences between predicted and experimental data would be higher, the higher the inner diameter of the employed HPLC column is.

In order to test this hypothesis, different temperature-gradient runs were performed and two of these runs were employed to calculate the values of S_T and k_0 for each analyte in the mixtures. On the basis of these two initial runs, retention times of the analytes were predicted for the other runs, which were not included in the data fitting process. Finally, predicted and experimental retention times were compared, depending on the inner diameter of the columns. Using this approach, the temperature-stability of the HPLC columns is a prerequisite. Otherwise, the application of high temperatures leads to a degradation of the packing material which concurrently results in a shift of retention times. Therefore, the high-temperature and pH stable silica-based Waters Bridged Ethylene Hybrid (BEH) and the metal oxide-based ZirChrom columns were employed for our study [42].

At first, temperature-gradient prediction based on interpolation will be discussed. Figure 2-2 a and b show a comparison of predicted vs experimental retention times of each steroid within a temperature range from 60 °C up to 160 °C. Predictions are based on experimental temperature gradients of 1.5 °C min^{-1} and 6.0 °C min^{-1}. The solid curves in Figure 2-2 a and b are the lines for $y = x$. Data points shown are obtained using temperature gradients with slopes of 2.5, 3.0, and 4.0 °C min^{-1}, which were not included in the data fitting process. Figure 2-2 a and b demonstrates that there are only minor differences between predicted and experimental retention times. This is true for both the 1 mm ID (Figure 2-2 a) and the 3 mm ID (Figure 2-2 b) column. The relative error ranges between 0.6% and 1% for the 1 mm ID column and between 0.6% and 1.6% for the 3 mm ID column. Moreover, the average relative error for predicted retention times using the 1 mm and 3 mm inner diameter column is 0.5%. In addition, if the root-mean-square error (RMSE) is considered, the retention times of the steroids can be predicted with an RMSE of 0.09 min using the 1 mm ID column and 0.11 min using the 3 mm ID column. These results point out that the difference between prediction and experimental data are independent from the diameter of the HPLC column.

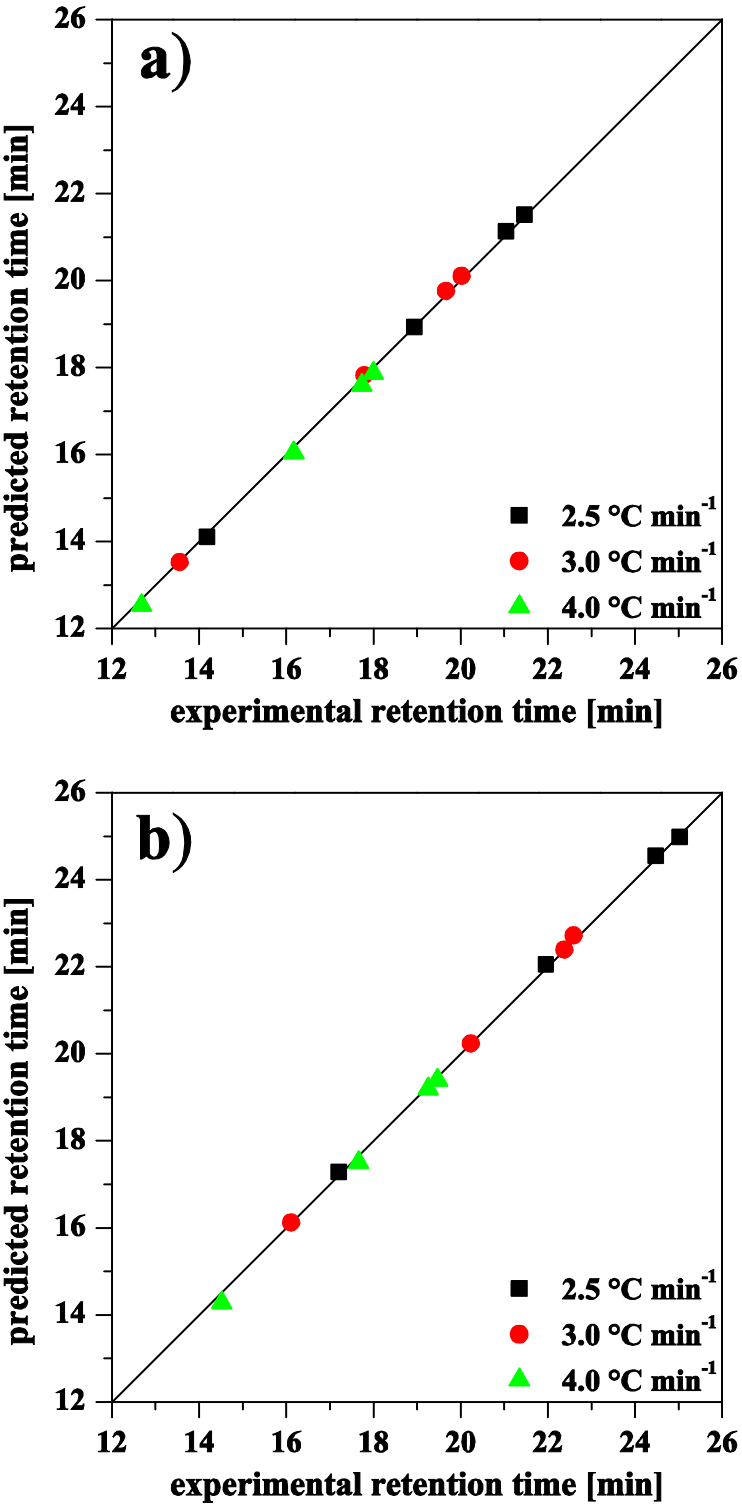

Figure 2-2: Predicted retention times calculated by LES vs experimental retention times of steroids. Predictions based on experimental runs of 1.5 °C min^{-1} and 6.0 °C min^{-1}. Temperature range: 60 °C - 160 °C; (a): ZirChrom-PBD (100 × 1.0 mm; 3 µm); root-mean-square error: 0.09 min; (b): ZirChrom-PBD (150 × 3.0 mm; 5 µm); root-mean-square error: 0.11 min; Elution order for every temperature gradient: 19-nortestosterone, testosterone, trans-dehydroandrosterone, and epitestosterone.

The steroid measurements shown above were carried out on two metal oxide-based stationary phases. Moreover, only temperature gradients with a maximal slope of 6.0 °C min^{-1} were investigated using a water mobile phase. In a further step, the LES approximation was evaluated using two silica-based Waters BEH stationary phases with diameters of 2.1 and 4.6 mm. The diameters of these columns were chosen to complete the range of column diameters which are usually employed in analytical HPLC. Furthermore, mobile phases which consisted of water and an organic modifier were investigated under isocratic conditions. In this case, the temperature range was set from 50 °C up to 180 °C. Within this range, significantly higher temperature-gradient slopes from 5 °C min^{-1} up to 30 °C min^{-1} were applied. In addition, in order to evaluate the LES model depending on the class of substances, for these experiments, a mixture of six PAHs was employed.

Figure 2-3 a and b show a comparison of predicted and experimental retention times of each PAH within the investigated temperature range. In Figure 2-3 a, a column with an inner diameter of 2.1 mm was employed and acetonitrile was used as organic modifier in the mobile phase. In contrast, in Figure 2-3 b, a column with an inner diameter of 4.6 mm was used and methanol was employed as organic co-solvent. Similar to Figure 2-2, the solid curves in Figure 2-3 a and b are the lines for $y = x$. In this case, the data fitting process was based on experimental gradients of 10 °C min^{-1} and 30 °C min^{-1}. The data points shown are obtained using temperature gradients with slopes of 12.5 °C min^{-1} up to 27.5 °C min^{-1} with an interval of 2.5 °C min^{-1}, which were not included in the data fitting process. Figure 2-3 a and b point out that there are only very small differences between predicted and experimental retention times of the PAHs. Furthermore, these differences are independent from the diameter of the employed column, which becomes also clear by the relative error. The maximal relative error ranges between 0.2% and 1.0% for the 2.1 mm ID column and between 0.6% and 1.1% for the 4.6 mm ID column. Moreover, an average error of 0.7% was calculated for the 2.1 mm ID column, whereas the average error for the 4.6 mm ID column is 0.4%. Also the RMSE of 0.03 min for the 2.1 mm ID and 0.02 min for the 4.6 mm ID column indicates the precision of retention time prediction using the LES model.

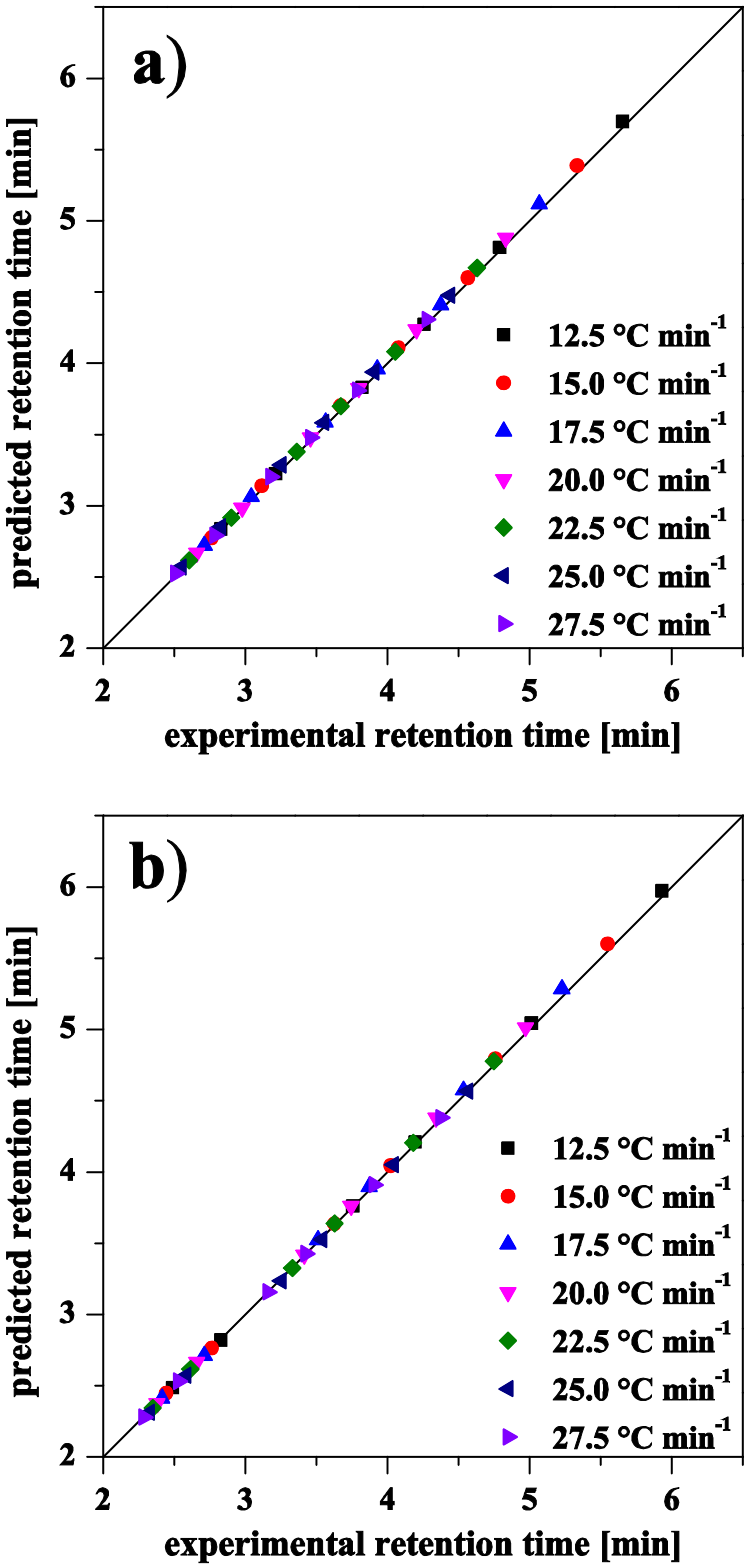

Figure 2-3: Predicted retention times calculated by LES vs experimental retention times of PAHs. Predictions based on experimental runs of 10 °C min^{-1} and 30 °C min^{-1}. Chromatographic conditions: temperature range: 50 °C - 180 °C; (a): Waters Acquity Phenyl (100 × 2.1 mm; 1.7 µm); root-mean-square error: 0.03 min; (b): Waters XBridge BEH C_{18} (75 × 4.6 mm; 2.5 µm); root-mean-square error: 0.02 min. Elution order for every temperature gradient: naphthalene, acenaphthylene, fluorene, anthracene, pyrene, and chrysene.

Figure 2-4 shows a comparison of a predicted (Figure 2-4 a) and the corresponding experimental chromatogram (Figure 2-4 b). In this case, a temperature gradient with a slope of 25 °C min^{-1} was applied on a Waters BEH column with an inner diameter of 4.6 mm. The peak width of the predicted chromatogram was calculated based on equations 10 and 14 of reference [26]. It can be seen that there are only marginal differences between the predicted and the experimental chromatogram. Furthermore, peaks were eluting symmetrically, and excessive peak band broadening due to radial temperature gradients could not be observed. The tailing factor at 10% peak height was between 0.9 and 1.2 which indicates a symmetric peak shape.

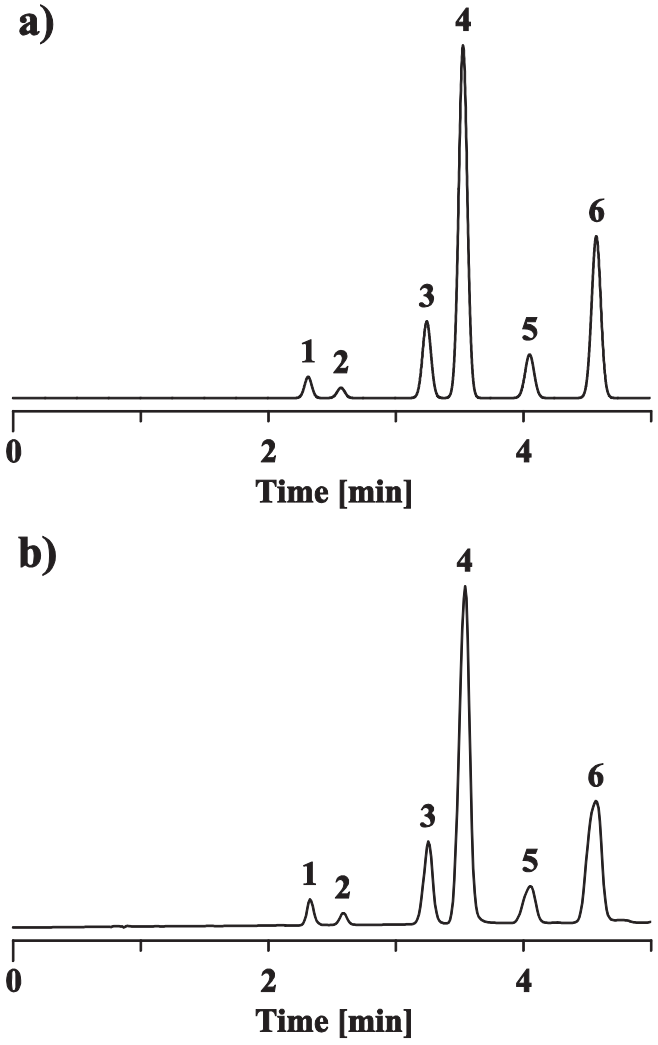

Figure 2-4: (a) Predicted chromatogram of PAHs, (b) experimental chromatogram of PAHs. Chromatographic conditions: temperature gradient: 50 °C - 180 °C in 5.2 min (25 °C min^{-1}); stationary phase: Waters XBridge C_{18} (75 × 4.6 mm; 2.5 µm). Analytes: 1: naphthalene; 2: acenaphthylene; 3: fluorene; 4: anthracene, 5: pyrene; 6: chrysene. For chromatographic conditions, see experimental section 2.3.4, PAH Mixture.

The predicted retention times of selected steroids and PAHs which are shown in Figure 2-2 and Figure 2-3 are based on interpolation. Next, it was investigated whether the LES approximation can also be employed to predict retention times on the basis of extrapolation of the input temperature-gradient runs. For the steroid mixture, the prediction starts with two experimental temperature-gradient runs with slopes of 1.5 °C min^{-1} and 3.0 °C min^{-1}. On the basis of these gradients the necessary model parameters were calculated and temperature-gradient runs of 4.0 °C min^{-1} and 6.0 °C min^{-1} were predicted. The results for both columns are shown in Table 2-1. It can be seen that there is a close agreement between predicted and experimental retention times for the 1 and 3 mm ID column with a maximal relative error of 1.5% and 1.8%, respectively. The relative errors shown in Table 2-1 have a negative sign, which points out that the extrapolated retention times of the steroids are usually smaller than the experimental values. This trend was not observed in the case of interpolated predictions.

Table 2-1: Comparison between Extrapolated Retention Times of Steroids calculated by LES vs Experimental Retention Times[a].

prediction [°C min^{-1}]	ID [mm]	analyte	exp. RT [min]	pred. RT [min]	error [min]	relative error [%]
4.0	1.0	19-nortestosterone	12.67	12.55	-0.12	-0.9
		testosterone	16.17	15.98	-0.19	-1.2
		trans-dehydroandrosterone	17.73	17.46	-0.27	-1.5
		epitestosterone	17.99	17.76	-0.24	-1.3
6.0	1.0	19-nortestosterone	11.02	11.03	+0.02	+0.1
		testosterone	13.52	13.45	-0.07	-0.5
		trans-dehydroandrosterone	14.62	14.46	-0.16	-1.1
		epitestosterone	14.82	14.69	-0.13	-0.9
4.0	3.0	19-nortestosterone	14.52	14.26	-0.26	-1.8
		testosterone	17.65	17.50	-0.15	-0.9
		trans-dehydroandrosterone	19.25	19.16	-0.10	-0.5
		epitestosterone	19.47	19.23	-0.24	-1.2
6.0	3.0	19-nortestosterone	11.80	11.77	-0.03	-0.3
		testosterone	14.03	14.01	-0.03	-0.2
		trans-dehydroandrosterone	15.20	15.10	-0.10	-0.1
		epitestosterone	15.28	15.15	-0.13	-0.1

[a] Prediction based on experimental temperature gradients of 1.5 °C min^{-1} and 3.0 °C min^{-1}. Temperature range: 60 °C - 160 °C.

Analogous to the steroid measurements, the possibility of extrapolations using the PAH mixture and the two silica-based Waters BEH columns was investigated. Therefore, experimental temperature gradients of 5 °C min^{-1} and 15 °C min^{-1} were employed to predict gradients with slopes of 17.5, 20.0, 22.5, 25.0, 27.5, and 30.0 °C min^{-1}. These results are shown in Figure 2-5 a and b for the 2.1 and 4.6 mm ID column, respectively. For the extrapolated retention times of the PAHs a relative error of 0.8% and 1.2% for the 2.1 mm and 4.6 mm ID column was observed, respectively. Because of these small errors it can be concluded that the LES approximation can also be employed in the case of extrapolation of the input runs.

Moreover, it can also be seen from Figure 2-5 a and b that the retention times of PAHs which were extrapolated by LES are slightly shifted to smaller retention times as the corresponding experimental values. However, this trend was also observed for the extrapolated retention times of the steroids. It is not unexpected that predictions based on extrapolations yield bigger errors when compared to predictions based on interpolation. It has to be considered that in both cases (steroids and PAHs) the slope of the extrapolated temperature gradients were doubled when compared with the input temperature-gradient runs.

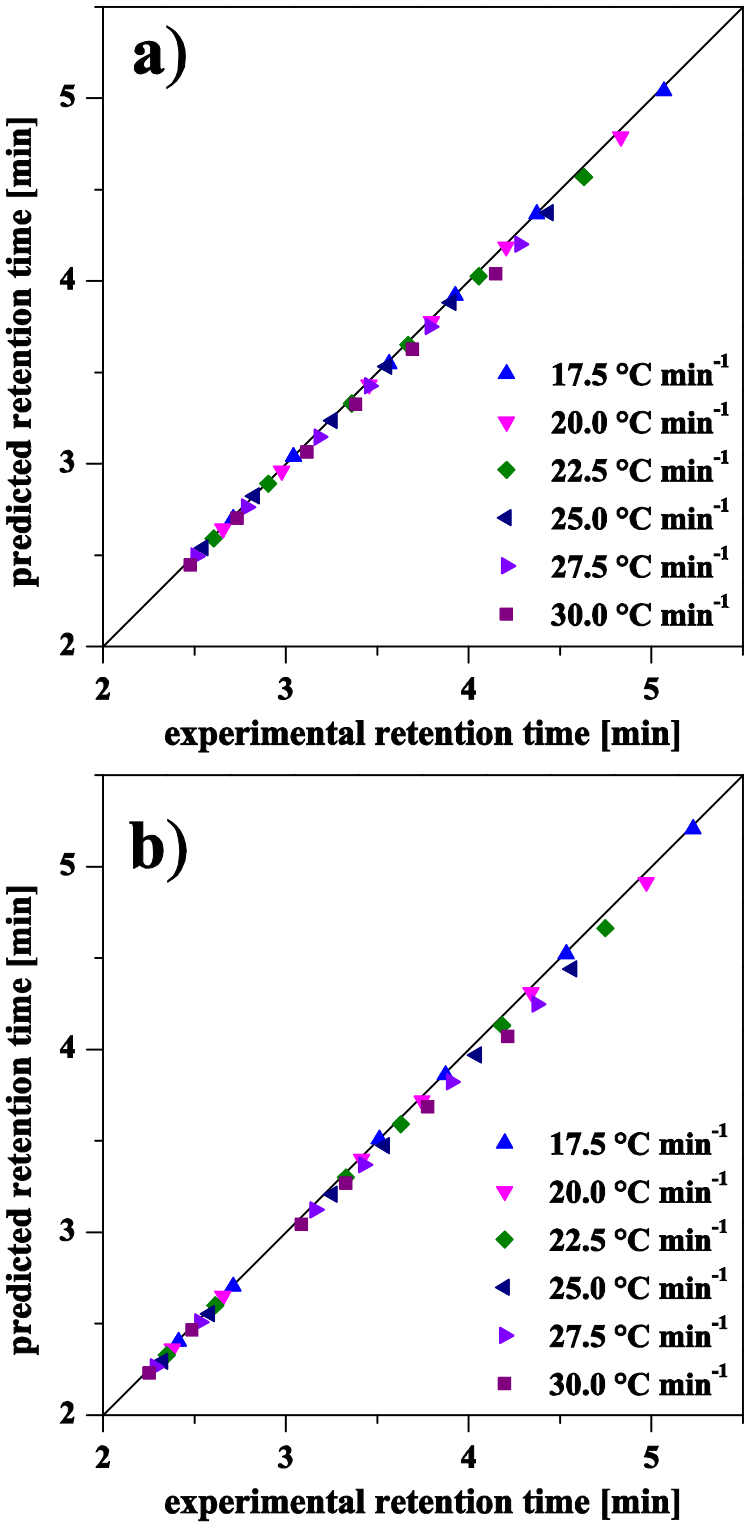

Figure 2-5: Predicted retention times calculated by LES vs experimental retention times of PAHs. Predictions based on experimental runs of 5 °C min^{-1} and 15 °C min^{-1}. Temperature range: 50 °C - 180 °C; (a): Waters Acquity Phenyl (100 × 2.1 mm; 1.7 µm); root-mean-square error: 0.04 min; (b): Waters XBridge BEH C_{18} (75 × 4.6 mm; 2.5 µm); root-mean-square error: 0.06 min. Elution order for every temperature gradient: naphthalene, acenaphthylene, fluorene, anthracene, pyrene, and chrysene.

Nevertheless, the observed trend in extrapolated retention times of steroids and PAHs could be interpreted as an argument that the heat transfer in the column does not take place immediately. If there would be a significant temperature-dependent delay, this time would be taken into account by the data fitting process. Hence, predictions based on interpolation should result in good correlation between prediction and experiment. In contrast, extrapolations to higher heating rates than those which were employed for the data fitting process should result in smaller retention times as the experimental retention times. In this case, the influence of the temperature-dependent delay time would be much higher. In this context, it is useful to compare our results with LES predictions in temperature-programmed GC. Bautz et al. [26] also performed retention time predictions based on extrapolations of two initial heating rates. The extrapolations were performed using a 30 m long GC column with an internal diameter of 250 µm. It was also observed that the extrapolated retention times predicted by the LES model were all lower than the experimental retention times (relative error: -1.2%). This trend could not be observed for interpolated values. If we take into account that in temperature-programmed GC the heat transfer occurs immediately because of the very small inner diameter of the GC columns (250 µm) it can be concluded that the errors observed are related to the extrapolation. This means that the errors in extrapolated retention times calculated by LES in HPLC are also related to the limitation of the extrapolation and not to a thermal lag as shown in Figure 2-1. Moreover, effects due to frictional heating can be excluded because the inlet pressure during all measurements in this study was less than 400 bar. Furthermore, a contribution of the effect of frictional heating regarding the accuracy of predicted retention times would be accounted for by the data fitting process.

Certainly, under temperature-gradient conditions in HPLC, radial temperature gradients will take place in the column. Our results show, however, that these radial temperature gradients and the related temperature-dependent delay time can be neglected for retention time predictions in temperature-programmed liquid chromatography although they may contribute to band broadening. Moreover, the results clearly indicate that the LES model can be used for method development in high-temperature liquid chromatography without mathematical extensions. This means that the heat at the outer side of the column is transferred very fast to the middle of the column packing so that the programmed temperature gradient closely matches the gradient that the analyte experiences in the column. This statement is in agreement with findings by Teutenberg where a prototype column oven based on the same concept as the SIM HT-HPLC 200 column oven was employed [43].

However, these results seem to be in contrast to findings by Nikitas and Pappa-Louisi [25]. They have introduced a two-mode gradient retention model which permits the simultaneous variation of mobile phase composition and temperature. Pappa-Louisi et al. reported an average relative error of 11.7% in predicted retention times using temperature gradients from 15 °C up to 75 °C (ΔT = 60 °C) under isocratic conditions (40% acetonitrile), where heating rates of 2.0, 3.0, 4.0, and 10.0 °C min^{-1} were investigated. In their study, a normal-bore column (150 × 4.6 mm, 3.5 µm) was used and the authors concluded that these errors are related to temperature hysteresis phenomena in the column oven and the column. To overcome this problem, they extended the model successfully by a differential equation to take the hysteresis phenomena into account. After that, an average error of 2.4% in predicted retention times was observed. In contrast, two significantly larger temperature intervals from 60 °C up to 160 °C (ΔT = 100 °C) and 50 °C up to 180 °C (ΔT = 130 °C) were employed in our study. Moreover, considerably higher heating rates from 1.5 °C min^{-1} up to 30 °C min^{-1} were investigated using high-temperature stable HPLC columns with inner diameters of 1.0, 2.1, 3.0, and 4.6 mm. Furthermore, in spite of the significant larger temperature intervals as well as the higher slopes of the temperature gradients, the observed average relative errors in predicted retention times of steroids and PAHs are less than 2% and comparable with the results presented by Nikitas and Pappa-Louisi. In general, differences in predicted vs experimental retention times should be small, although errors up to 5% are acceptable [27].

The mathematical extension of the retention model regarding a temperature-dependent delay time as described in the work of Nikitas and Pappa-Louisi is necessary because of the heating system. Pappa-Louisi and Nikitas employed a conventional HPLC air-bath column oven for their measurements. Thus, the heat transfer takes place between the heated air and the outer metal surface of the HPLC column. In our study, a specially designed high-temperature column oven was used [41]. In this case, the heat transfer is obtained by block heating where the column is tightly enclosed between two aluminum shells which are connected with the heating block. Hence, if an air bath column oven is used and temperature gradients are employed, a temperature-dependent delay time has to be considered for the prediction of retention times.

2.5 Conclusion

In general, the LES model can be used to predict temperature gradients in LC based on two initial runs, but this is only true if a high-temperature column oven, based on the block heating concept is used. Moreover, precise predictions with an average relative error < 2% using a water mobile phase as well as binary mixtures which consist of water and an organic co-solvent such as methanol or acetonitrile under isocratic conditions can be made. Furthermore, method development is not restricted to a certain inner diameter of the HPLC column which enables the use of columns with diameters from at least 1.0 mm up to 4.6 mm. Nevertheless, a critical point is the LES approximation in temperature-programmed LC, because predictions based on this model are restricted to the experimental conditions. This means the predictions are only adequate if the following requirements are met. First, the composition of the mobile phase is equal to the composition of the mobile phase which was employed for the input runs. Second, the start temperature of the predicted temperature-gradient runs is equal to the start temperature of the input runs. In a further publication, we will describe how some of these limitations can be overcome.

Acknowledgment

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2.6 References

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Chapter 3. A General Strategy for Performing Temperature Programming in High Performance Liquid Chromatography – Prediction of Segmented Temperature Gradients*

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3.1 Introduction

In recent years some high-temperature liquid chromatography (HT-HPLC) based hyphenation techniques [1-13] have been developed. These techniques require the use of a water mobile phase or the content of organic solvent in the mobile phase being as low as possible. In contrast to other approaches where temperature is usually discussed in terms of speeding up a separation [14,15] or increasing the efficiency [16,17], these hyphenation techniques make use of the fact that increasing the temperature of water results in a decrease in its static permittivity [18]. In other words, the higher the temperature of water, the lower the polarity of a water mobile phase. Therefore, whenever a mixture has to be separated where the polarity of the compounds is not too broad, temperature gradients can be employed instead of solvent gradients as has been shown elsewhere [7,19-21]. Hence, the practitioner is faced with the problem how to develop a method where temperature gradients are employed instead of solvent gradients. Most attempts to achieve adequate separations in temperature-programming mode under isocratic or solvent gradient conditions are governed by trial and error [22-25], which also owes to the lack of sufficient prediction models. To overcome this problem, Nikitas and Pappa-Louisi developed retention models which permit prediction of retention times when solvent composition and temperature are changed simultaneously [26-28]. To date, 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 small temperature range from 15 °C up to 75 °C (ΔT = 60 °C).

In a recent study [29] we could show that the linear elution strength (LES) model from temperature-programmed gas chromatography (GC) can be employed for retention time predictions for linear temperature gradients in temperature-programmed liquid chromatography (LC) under isocratic conditions. It was shown that retention times for temperature gradients with slopes up to 30 °C min^{-1} in a temperature range from 50 °C up to 180 °C can be predicted with high accuracy when the start temperature of the gradient is not changed. On the basis of these findings the aim of this work was to extend this model in order to predict retention times for more complex segmented temperature gradients in LC under isocratic conditions and to evaluate the errors in predicted retention times when the start temperature of the gradient is changed.

In high-temperature LC, the practitioner has the choice between an isothermal or a temperature-gradient operation mode. In order to reduce the experimental work during

method development, it will be advantageous when only one basic data set is used for isothermal as well as temperature-gradient method development. In this context, the generation of temperature-gradient input data is less time consuming and requires less experimental work when compared to isothermal data acquisition. Moreover, in temperature-programming mode, samples containing analytes with different polarities can be measured within the same chromatographic run. In isothermal operation mode, the less polar compounds of the sample mixture will be retarded strongly at low temperatures, giving rise to very long retention times. Therefore, it would be advantageous if it is possible to predict isothermal as well as temperature-gradient separations based only on temperature-gradient input data, despite the fact that isothermal input data might be more precise and less affected by linear retention assumptions than temperature-gradient input data [30]. Moreover, systematic method development of an isothermal as well as temperature-gradient separation of selected sulfonamides will be performed based on as few input measurements as possible. Finally, both operation modes will be compared.

3.2 Experimental Section

3.2.1 Chemicals

A test mixture of five sulfonamides consisting of sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfamethoxazole, and uracil was used. The analytes were dissolved in 75/25 (v/v) water/acetonitrile at a concentration of 50 µg mL^{-1}. Formic acid was employed to adjust the pH of the mobile phase to 2.7. All chemicals given above were purchased from Sigma-Aldrich (Seelze, Germany) in p. a. quality and used without further purification. High-purity water was prepared by an Elix 10-Milli-Q Plus water purification system (Millipore, Eschborn, Germany). Acetonitrile (ACN) was purchased from LGC Standards (Wesel, Germany) in Optigrade quality.

3.2.2 HPLC System

A Shimadzu HPLC system (Shimadzu, Duisburg, Germany) was used which consists 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 back pressure 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.

3.2.3 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 specially 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. In order to compensate effects which are related to frictional heating, the practitioner can define a temperature difference between the eluent preheating and the column heating unit. 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 settings of the preheating unit and the column were identical.

3.2.4 Isothermal/Isocratic Measurements

The measurements were carried out on a Waters XBridge C_{18} (75 × 4.6 mm; 2.5 µm) column using a water mobile phase with 0.1% formic acid at a flow rate of 1.0 mL min^{-1}. For isothermal runs at 60 °C and 80 °C the injection volume was set to 2 µL whereas for measurements from 100 °C up to 180 °C the injection volume was set to 1 µL. UV detection was carried out at a wavelength of 270 nm. The sampling rate and the time constant of the UV detector were set to 3.125 Hz and 0.32 sec, respectively.

3.2.5 Temperature-Gradient Measurements

Two temperature ranges from 60 °C up to 180 °C and 100 °C up to 180 °C were investigated. Temperature gradients with slopes from 2 °C min^{-1} up to 12 °C min^{-1} were applied. All other chromatographic conditions equal the respective isothermal measurements.

3.3 Theory

In a previous study [29] we have shown that the LES model could successfully be adapted from temperature-programmed gas chromatography to temperature-programmed liquid chromatography. Moreover, it was shown that it is not necessary to extend the LES model to consider a temperature-dependent delay time when a high-temperature column oven based on block heating is employed. Using the LES model the retention time t_R of an analyte can be predicted as a function of experimental conditions using equations 3-1 and 3-2 [33,34].

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

$$\text{with } b_T = \frac{t_0 S_T \Delta T}{tG} \tag{3-2}$$

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. 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 time 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 equations 3-1 and 3-2. This procedure is very similar to numerical solutions of the LSS relationship [36-38]. An example of how a spreadsheet calculator can be used for the numerical solution is given in the Appendix for Chapter 3.

Regarding the estimation of the column dead time t_0 usually the retention time of an unretained compound such as uracil is used [39]. However, the column dead time can also be approximated by equation 3-3 [40].

$$t_0 \approx 5 \times 10^{-4} \frac{L d_c^2}{F} \tag{3-3}$$

Here L is the column length in mm, d_c is the column inner diameter in mm, and F is the flow rate in mL min^{-1}. In this study the column dead time was calculated according to equation 3-3 by using a factor of 4.85×10^{-4} instead of 5×10^{-4}.

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 this case, a similar derivation to solvent gradient elution yields [33,41,42].

$$t_R = \frac{t_0}{2.3 b_T} \ln\left[e^{2.3 b_T r} \left(k_0 + 1\right) - k_0 \right] \tag{3-4}$$

Furthermore, in the case where a temperature-gradient method consists of an isothermal/isocratic hold, equation 3-5 can be employed to calculate the retention time of an analyte during this segment.

$$t_R = r t_0 \left(k_0 + 1\right) \tag{3-5}$$

The sum of the fractional migration r of an analyte across the column during each temperature segment can also be written as $(r_1 + r_2 + ... r_n = 1)$. Moreover, equation 3-6 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 employed instead of k_0 in equation 3-4.

$$\log k_r = \log k_0 - \frac{b_T t_R}{t_0} \tag{3-6}$$

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 Appendix for Chapter 3.

In order to predict isothermal retention times based on temperature gradients, the LES approximation assumes that there is a relationship between the retention factor of the analyte in temperature gradient and in isothermal elution [33]. Hence, equation 3-7 is employed to predict isothermal retention times of the analytes based on two temperature-gradient runs in temperature-programmed GC, where A' represents an analyte specific constant and k_i describes the isothermal retention factor of the solute i.

$$\log k_i = A' - S_T T \tag{3-7}$$

Moreover, equation 3-7 is used to calculate the retention factor of an analyte when the start temperature of the temperature gradient for which the retention factors are being predicted is different from the start temperature of the gradients which have been employed during data fitting.

The described simplification in equation 3-7 is very similar to solvent gradient elution, where isocratic retention times can be approximated based on two solvent gradient input runs [43,44]. Nevertheless, the approach to predict isothermal retention times based on temperature-gradient data has not been used frequently, because usually the van't Hoff relationship is employed to predict isothermal data in chromatography which is given as

$$\ln k_i = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \beta \tag{3-8}$$

where T is the column temperature in K, k_i refers to the retention factor of the solute i, ΔH and ΔS are the enthalpy and entropy of transfer of the solute i from the mobile into the stationary phase, R is the ideal gas constant and β is the volume phase ratio of the stationary and mobile phase. Moreover, the van't Hoff equation assumes that the enthalpy and entropy of transfer and the volume phase ratio are independent from temperature [45-47]. It should be noted that equations 3-7 and 3-8 distinguish between the usually employed input data. Equation 3-7 makes use of temperature-gradient data whereas isothermal data are employed using equation 3-8.

3.4 Results and Discussion

3.4.1 Isothermal Predictions based on Temperature-Gradient Input Data

Method development always targets a separation with a user-defined resolution as fast as possible. In high-temperature liquid chromatography the user has the choice between an isothermal and temperature-programming mode to achieve a suitable separation. As pointed out before, under certain conditions method development based on temperature-gradient measurements provides some advantages. Therefore, the accuracy of isothermal retention time predictions based on temperature-gradient input runs was investigated first.

Regarding the LES model, predictions of isothermal retention times in GC can be performed based on two temperature-gradient runs [33,34,48]. Afterwards, equation 3-7 was employed to calculate the corresponding isothermal separations. Analogous to isothermal predictions in temperature-programmed GC, the feasibility to make use of equation 3-7 was investigated to predict isothermal/isocratic separations in temperature-programmed LC. For this approach, the sulfonamide test mixture was employed and a temperature range from 60 °C up to 180 °C was chosen. Within this range two temperature gradients were measured on a Waters XBridge C_{18} column. Afterwards, the retention data of each analyte from these two temperature gradients were employed to predict isothermal/isocratic retention factors at 60, 80, 100, 120, 140, 160, and 180 °C. The results of these isothermal retention factor predictions are shown in Table 3-1.

As can be seen, significant deviations between predicted and experimental retention factors are observed. The relative error for predicted retention factors is between 2.9% and 169.6%. Moreover, Table 3-1 points out that calculations based on equation 3-7 usually yield retention factors which are much higher than the experimental values, except for a temperature of 60 °C which is the start temperature of the basic temperature-gradient measurements. If retention times are calculated for a temperature of 60 °C, the average error is 4.3%. Very similar results were obtained using selected polycyclic aromatic hydrocarbons as model compounds, different columns and different mobile phases consisting of water and methanol as well as acetonitrile (data not shown here).

Table 3-1: Comparison of predicted vs experimentally obtained retention factors of selected sulfonamides.

temperature [°C]	analyte	$k_{0,\ predicted}$ [a, b]	$k_{0,\ experimental}$ [b]	difference of k_0	relative error [%]
60	sulfadiazine	11.16	10.57	0.59	5.6
	sulfathiazole	16.08	15.55	0.53	3.4
	sulfamerazine	28.68	27.47	1.21	4.4
	sulfamethoxazole	61.29	65.39	4.10	6.3
	sulfamethazine	71.29	69.92	2.00	2.9
80	sulfadiazine	8.28	6.61	1.67	25.3
	sulfathiazole	10.68	8.51	2.17	25.5
	sulfamerazine	19.69	17.00	2.69	15.8
	sulfamethoxazole	36.83	33.75	3.08	9.1
	sulfamethazine	46.34	42.36	3.98	9.4
100	sulfadiazine	6.14	4.26	1.88	44.1
	sulfathiazole	7.09	4.86	2.23	45.9
	sulfamerazine	13.51	10.68	2.83	26.5
	sulfamethoxazole	22.13	18.09	4.04	22.3
	sulfamethazine	29.86	25.96	3.90	15.0
120	sulfadiazine	4.56	2.74	1.82	66.4
	sulfathiazole	4.71	2.74	1.97	71.9
	sulfamerazine	9.28	6.53	2.75	42.1
	sulfamethoxazole	13.30	9.69	3.61	37.3
	sulfamethazine	19.24	15.30	3.94	25.8
140	sulfadiazine	3.38	1.70	1.68	98.8
	sulfathiazole	3.13	1.60	1.53	95.6
	sulfamerazine	6.37	3.96	2.41	60.9
	sulfamethoxazole	7.99	5.29	2.70	51.0
	sulfamethazine	12.40	8.95	3.45	38.5
160	sulfadiazine	2.51	1.09	1.42	130.3
	sulfathiazole	2.08	0.95	1.13	118.9
	sulfamerazine	4.37	2.44	1.93	79.1
	sulfamethoxazole	4.80	2.97	1.83	61.6
	sulfamethazine	7.99	5.25	2.74	52.2
180	sulfadiazine	1.86	0.69	1.17	169.6
	sulfathiazole	1.38	0.56	0.82	146.4
	sulfamerazine	3.00	1.49	1.51	101.3
	sulfamethoxazole	2.88	1.71	1.17	68.4
	sulfamethazine	5.15	3.10	2.05	66.1

[a] Predictions based on experimental temperature-gradient measurements from 60 °C to 180 °C within 20 (6 °C min^{-1}) and 60 (2 °C min^{-1}) minutes according to equation 3-7.

[b] For calculations a column dead time of 0.77 minutes was used.

In temperature-programmed GC Bautz and co-workers concluded that isothermal retention time predictions based on two temperature gradients can be performed with an error smaller than 5% within a temperature range from 50 °C up to 250 °C where temperature gradients with slopes from 1 °C min^{-1} up to 12 °C min^{-1} were used [33,34,48,49]. These results are in contrast to our findings. However, they pointed out that significant errors in predicted isothermal retention times can arise from extrapolation of equation 3-7 with regard to the considered retention factor range. Furthermore, Nawas and Poole [50] also investigated the possibility to predict isothermal retention times based on two temperature gradients in a temperature range from 60 °C to 160 °C in temperature-programmed GC. In their study temperature gradients with slopes of 3 °C min^{-1} and 12 °C min^{-1} were employed to predict isothermal retention times in the range from 60 °C up to 140 °C. They concluded that the linear elution strength model is unsuitable to predict isothermal retention times of solutes over a wide temperature range. It should be noted that the studies cited above were carried out in temperature-programmed GC, whereas in our study the LES approximation is employed in temperature-programmed LC. One could think that thermal mass effects or thermal lag phenomena might be responsible for the observed major relative errors of predicted retention times, because a 4.6 mm ID column has been used in this study. It should be considered, however, that a specially designed high-temperature column oven was employed which assures that the heat at the outer side of the column is transferred very quickly to the column packing. Therefore, the applied temperature gradient closely matches the gradient that the analyte experiences in the column. This was also experimentally confirmed by Teutenberg et al. for a column with an inner diameter of 4.6 mm [31,51], which is in complete agreement with our experimental results given in reference [29].

Therefore, it becomes obvious that isothermal predictions based on temperature-gradient input data, calculated by means of equation 3-7 will not give accurate results in temperature-programmed LC. This also restricts the ability to predict temperature-gradients in LC, where the start temperature of the predicted gradient differs from the start temperature of the gradients which have been employed during data fitting. This means, if the data fitting was carried out using temperature gradients from 60 °C up to 180 °C the same start temperature of 60 °C as well as the same composition of the mobile phase have to be chosen for method development.

Hence, in order to overcome the restriction regarding the start temperature of the temperature gradient, a mathematical expression is necessary which describes the dependence of the retention factor of a solute on temperature at a constant mobile phase composition. From the point of structured method development in temperature-programmed LC this means that the van't Hoff equation should be employed to describe the relationship between retention and temperature. If linearity is assumed, at least two additional isothermal/isocratic input runs are necessary.

3.4.2 Isothermal Predictions based on Isothermal Input Data

For these predictions the sulfonamide test mixture was employed and a temperature range from 60 °C up to 180 °C was chosen. Within this range a van't Hoff analysis was carried out with a temperature increment of 20 °C at a flow rate of 1.0 mL min^{-1} using a water mobile phase with 0.1% formic acid. Figure 3-1 shows the van't Hoff plots which are based on these measurements.

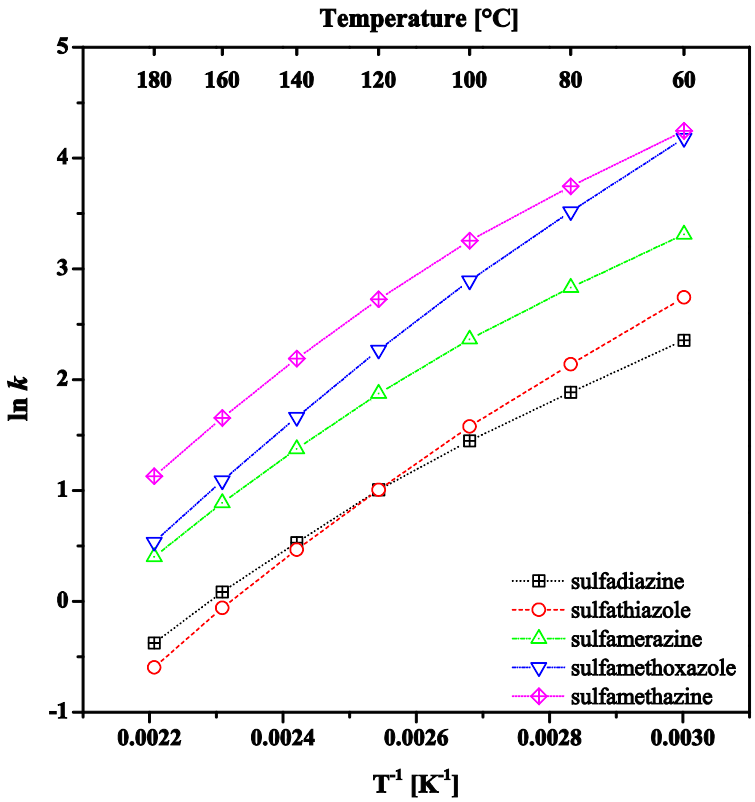

Figure 3-1: Van't Hoff plot of selected sulfonamides in a temperature interval from 60 °C to 180 °C. Chromatographic conditions: stationary phase: Waters XBridge C_{18} (75 × 4.6 mm, 2.5 µm); mobile phase: deionized water with 0.1% formic acid; flow rate: 1.0 mL min^{-1}; injection volume: 2 µL at 60 °C and 80 °C, 1 µL from 100 °C to 180 °C; detection: UV at 270 nm. Coefficient of determination of linear regression (R^2): sulfadiazine = 0.9915, sulfathiazole = 0.9955, sulfamerazine = 0.9908, sulfamethoxazole = 0.9963, sulfamethazine = 0.9893.

It can be seen that each sulfonamide exhibits a curved van't Hoff plot over the whole temperature range whereas theoretically a straight line was expected. Furthermore, deviations of the expected linear behavior of the van't Hoff plot of each sulfonamide are underlined by the coefficient of determination (R^2) of linear regression and depicted in the figure caption. Moreover, it can also be seen that the elution order of sulfathiazole and sulfadiazine is

changed at a temperature of approximately 120 °C underlining that temperature can be employed to change selectivity in LC [17,52].

In the scientific literature, linear [53,54] as well as non-linear van't Hoff plots [55,56] are described using different mobile and stationary phases and analytes. The scope of our study, however, was not to investigate why non-linear van't Hoff plots were observed but rather how this non-linear plots of $\ln k$ vs $1/T$ can be handled during structured method development in high-temperature liquid chromatography. Usually, two isothermal/isocratic runs are employed for linear regression of the $\ln k$ vs $1/T$ plot which will then be used to predict isothermal retention factors. Related to our isothermal/isocratic sulfonamide measurements, a linear regression of the $\ln k$ vs $1/T$ plot was carried out using retention data from 60 °C and 180 °C to predict retention times of the analytes at temperatures of 80, 100, 120, 140, and 160 °C. The relative errors between predicted and experimental retention times of the sulfonamides are shown in Table 3-2 and make clear that this approach yields errors as large as 23%.

Table 3-2: Comparison of relative errors between predicted and experimental retention times of selected sulfonamides calculated by linear regression of the van't Hoff plot using two isothermal/isocratic runs at 60 °C and 180 °C.

	relative error [%] at selected temperatures				
analyte	**80 °C**	**100 °C**	**120 °C**	**140 °C**	**160 °C**
sulfadiazine	–9.5	–14.6	–14.8	–10.0	–5.5
sulfathiazole	–9.4	–14.1	–12.6	–9.4	–5.0
sulfamerazine	–12.5	–19.0	–18.6	–14.0	–7.6
sulfamethoxazole	–10.8	–16.4	–15.7	–11.5	–6.3
sulfamethazine	–14.9	–22.8	–22.6	–17.9	–10.0
average error [%]	11.4	17.4	16.9	12.6	6.9

In other words, when a large temperature interval such as 120 °C is studied and non-linear van't Hoff plots are observed, isothermal retention time predictions based on linear regression of the $\ln k$ vs $1/T$ plot are unsuitable during method development in high temperature LC. There are some options to overcome this problem. First it is possible to reduce the temperature range, e.g. to $\Delta T = 40$ °C or 50 °C, because then a linear regression of the $\ln k$ vs $1/T$ plot should yield a more reliable isothermal prediction. Regarding our measurements, a decrease of the considered temperature range from $\Delta T = 120$ °C (60 °C - 180 °C) to $\Delta T = 40$ °C (60 °C - 100 °C) results in a significant improvement in the

accuracy of predicted retention times. In this case a maximal relative error of 2.2% was observed.

Another option is the use of mathematical expressions which are able to describe curved plots of $\ln k$ vs $1/T$. In this context Pappa-Louisi and Nikitas could show that other equations can be successfully employed to fit curved $\ln k$ vs $1/T$ plots where only measurements at two temperatures were necessary [28]. They also noted that when a model is used which predicts curved plots of $\ln k$ vs $1/T$ under isocratic conditions, the operator should carefully check that no problems arise from overfitting the experimental data [28]. From a practical point of view, this means that additional runs are required so that the user can be sure that the data fitting was successful.

However, it is also possible to consider a plot of $\ln k$ vs T [50], which is shown in Figure 3-2.

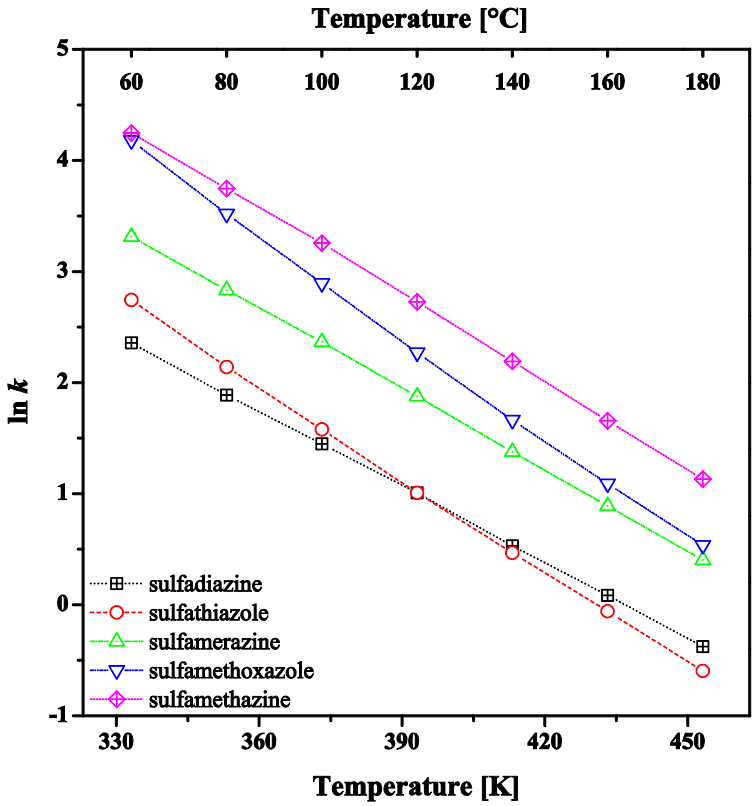

Figure 3-2: Plot of $\ln k$ vs T temperature of selected sulfonamides in a temperature range from 60 °C to 180 °C. Chromatographic conditions: stationary phase: Waters XBridge C_{18} (75 × 4.6 mm, 2.5 µm); mobile phase: deionized water with 0.1% formic acid; flow rate: 1.0 mL min^{-1}; injection volume: 2 µL at 60 °C and 80 °C, 1 µL from 100 °C to 180 °C; detection: UV at 270 nm. Coefficient of determination of linear regression (R^2): sulfadiazine = 0.9999, sulfathiazole = 0.9995, sulfamerazine = 0.9999, sulfamethoxazole = 0.9992, sulfamethazine = 0.9997.

It can be seen that all sulfonamides exhibit a linear plot of $\ln k$ vs T, which is also underlined by the coefficient of determination of linear regression and depicted in the figure caption for each analyte. Analogous to the van't Hoff plot a linear regression was carried out using the retention data from 60 °C and 180 °C based on the plot of $\ln k$ vs T. Afterwards, retention times of all analytes at temperatures of 80, 100, 120, 140 and 160 °C were predicted. Subsequently, the predicted and experimentally obtained retention times were compared. The relative error was calculated and is represented in Table 3-3.

Table 3-3: Comparison of relative errors between predicted and experimental retention times of selected sulfonamides calculated by linear regression of the $\ln k$ vs T plot using two isothermal/isocratic runs at 60 °C and 180 °C.

	relative error [%] at selected temperatures				
analyte	**80 °C**	**100 °C**	**120 °C**	**140 °C**	**160 °C**
sulfadiazine	−1.2	+0.3	+1.3	−0.3	+0.3
sulfathiazole	−4.2	−4.4	−5.0	−3.2	−1.0
sulfamerazine	+0.5	+2.2	+1.6	+0.3	+0.1
sulfamethoxazole	−5.4	−6.9	−8.4	−7.7	−4.1
sulfamethazine	+1.8	+4.5	+3.5	+1.9	+0.6
average error [%]	2.6	3.7	4.0	2.7	1.2

It can be concluded that the predicted retention times are now in agreement with the experimental data. Isothermal/isocratic retention times of the sulfonamides can be predicted with a relative error of less than 5% except for sulfamethoxazole where a maximal relative error of 8.4% was observed at 120 °C. Compared to predictions based on the van't Hoff analysis (see Table 3-2), predictions using a plot of $\ln k$ vs T obviously yield more accurate isothermal retention times. However, based on the results shown above, a strictly linear relationship between $\ln k$ and T cannot be assumed for all possible analytes. For systematic method development it would be advantageous to perform three isothermal measurements. Afterwards, the user is able to choose a plot and/or a mathematical expression where the error between prediction and experiment is as low as possible.

Because of the results shown in Figure 3-2 and Table 3-3 and in order to underline that a plot of $\ln k$ vs T can be a useful tool, linear regressions of the $\ln k$ vs T plot have been employed for isothermal method development. This approach will be described exemplarily for the isothermal separation of the sulfonamide mixture. Considering Figure 3-2 there are two temperature ranges where an isothermal separation of the sulfonamides will be possible,

extending from 70 °C to 100 °C and from 150 °C to 180 °C. This becomes also clear by the experimental chromatograms shown in Figure 3-3.

At a temperature of 60 °C (Figure 3-3 a) the separation of the first three sulfonamides is very good with a resolution (R) higher than 5.8 whereas the critical resolution (R_S) between sulfamethoxazole and sulfamethazine (peak pair 5/6) is inadequate ($R_S = 0.9$). Furthermore, at this temperature a long analysis time of approximately 60 minutes can be observed, and the last peaks are eluted as broad bands. Increasing the temperature to 80 °C (Figure 3-3 b), the analytes can now be baseline separated within approximately 36 minutes with a critical resolution between sulfadiazine and sulfathiazole (peak pair 2/3) of 4.0. In order to shorten the analysis time, the temperature can be increased further. For example, at a temperature of 120 °C (Figure 3-3 d) the resolution between peak pair 5/6 is now very high ($R = 7.2$), whereas sulfadiazine and sulfathiazole completely co-elute. At this temperature, however, the analysis time was reduced to approximately 14 minutes. If the temperature is increased even further to e.g. 180 °C (Figure 3-3 g) an analysis time of about 3.5 minutes can be obtained. Moreover, all sulfonamides were separated with a critical resolution of 1.1. This might be appropriate for UV detection but is insufficient for the special hyphenation techniques mentioned in the introduction. The reason is that these techniques require a baseline separation of the analytes. In other words, the critical resolution must exceed 1.5. Moreover, often the analytes cannot be dissolved in water and a certain organic content is necessary. This means that the injected sample plug might contain a high amount of an organic solvent which results in a very strong solvent peak at the beginning of the chromatogram. The tailing of the solvent peak can extend over 5 to 10 minutes and the early eluting peaks elute on the tailing of the solvent peak. To overcome this issue, a method where the first analyte elutes after several minutes is needed. Moreover, it has to be considered that the column lifetime can be significantly increased if the separation is performed at 80 °C when compared to a separation at 180 °C. Therefore, the isothermal separation of the sulfonamides at 180 °C (Figure 3-3 g) is rather unsuitable when considering both column lifetime and the requirements of special hyphenation techniques.

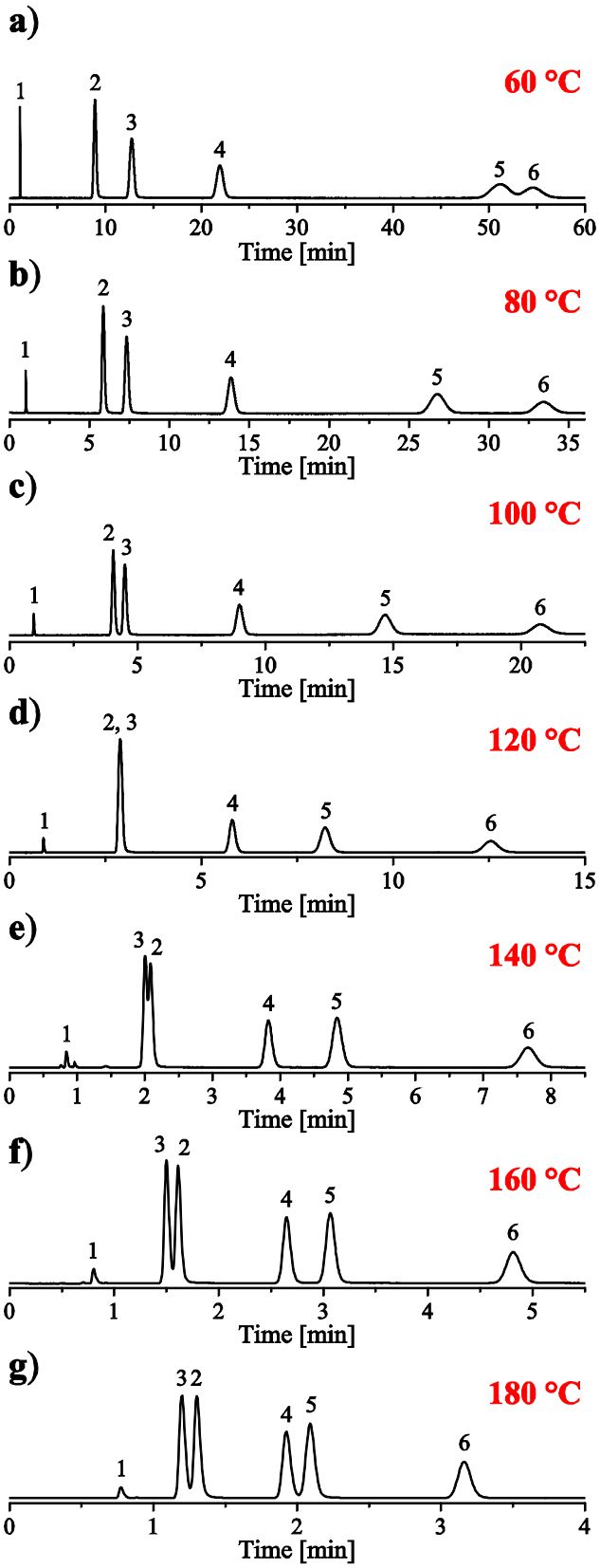

Figure 3-3: Isothermal separations of five sulfonamides and uracil. Chromatographic conditions: stationary phase: Waters XBridge C_{18} (75 × 4.6 mm, 2.5 µm); mobile phase: deionized water with 0.1% formic acid; flow rate: 1.0 mL min^{-1}; injection volume: 2 µL at 60 °C and 80 °C, 1 µL from 100 °C to 180 °C; detection: UV at 270 nm. Analytes: 1) uracil, 2) sulfadiazine, 3) sulfathiazole, 4) sulfamerazine, 5) sulfamethoxazole, 6) sulfamethazine.

3.4.3 Temperature-Gradient Predictions based on Temperature-Gradient Runs and Isothermal Measurements

The other option to achieve a good separation of the sulfonamide mixture will be the use of more complex segmented temperature gradients. Complex means that the temperature gradients consist of gradients with different slopes or a combination of temperature gradients and isothermal holds. The temperature-gradient method development starts with two initial temperature-gradient runs to calculate the required LES model parameters S_T and k_0 for each sulfonamide in the desired temperature range. In this case a temperature range from 60 °C up to 180 °C was chosen. Within this range two temperature gradients were applied with slopes of 2 °C and 6 °C min^{-1}. Afterwards, the model parameters S_T and k_0 for each sulfonamide were calculated. In the case of simple linear temperature gradients with different gradient slopes equations 3-1 and 3-2 are employed. In the case of more complex segmented temperature gradients, equations 3-4 to 3-6 are also needed to calculate the fractional migration of the solutes across the column during each temperature segment. On the basis of the LES model, two methods were developed for the separation of the sulfonamides which are shown in Figure 3-4.

The first method has a start temperature of 60 °C and consists of two linear temperature gradients with slopes of approximately 12 °C min^{-1} and two isothermal holds at 115 °C and 180 °C, the latter temperature corresponding to the upper temperature limit. The separation of the sulfonamides was performed within 13 minutes with a critical resolution of 3.4 between sulfadiazine and sulfathiazole. In order to demonstrate how well retention times can be simulated for segmented temperature gradients in LC, Table 3-4 shows a comparison between predicted and experimental retention times of the sulfonamides with a maximal relative error of 4.3% (average relative error: 2.2%). Moreover, it has to be considered that method development is based on only two temperature-gradient basic measurements. A detailed calculation of predicted retention times of the sulfonamides shown in Figure 3-4 is given in the Appendix for Chapter 3.

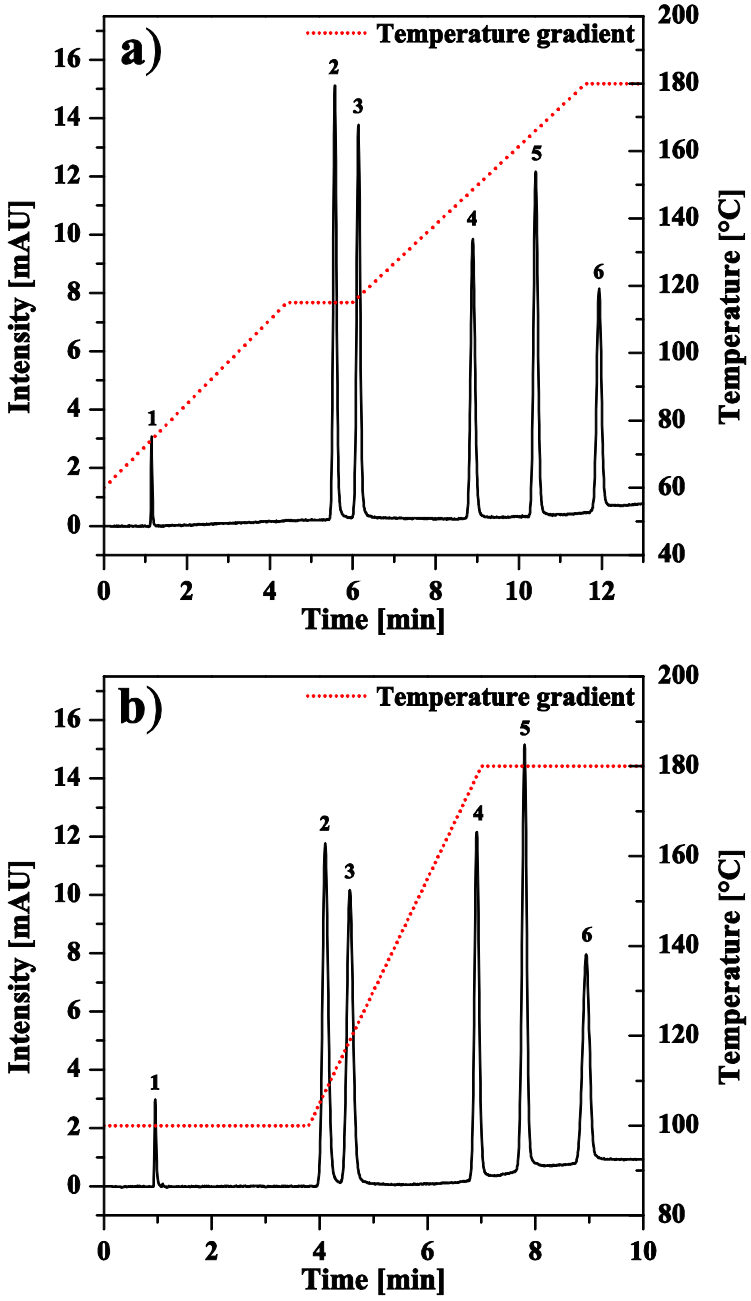

Figure 3-4: Separation of five sulfonamides and uracil by temperature gradient elution. a) start temperature: 60 °C, b) start temperature 100 °C. Chromatographic conditions: stationary phase: Waters XBridge C_{18} (75 × 4.6 mm, 2.5 µm); mobile phase: deionized water with 0.1% formic acid; flow rate: 1.0 mL min^{-1}; temperature gradient: see Figure 3-4; injection volume: 1 µL; detection: UV at 270 nm. Analytes: 1) uracil, 2) sulfadiazine 3) sulfathiazole, 4) sulfamerazine, 5) sulfamethoxazole, 6) sulfamethazine.

Table 3-4: Comparison of predicted retention times calculated by LES model and experimental retention times of sulfonamides.

figure	analyte	expt. RT [min]	pred. RT [min]	difference [min]	relative error [%]
3-4 a	sulfadiazine	5.56	5.80	+0.24	+4.3
	sulfathiazole	6.13	6.38	+0.25	+4.1
	sulfamerazine	8.89	9.07	+0.18	+2.0
	sulfamethoxazole	10.41	10.40	–0.01	–0.1
	sulfamethazine	11.93	12.01	+0.08	+0.6
3-4 b	sulfadiazine	4.10	4.03	–0.07	–1.7
	sulfathiazole	4.56	4.57	+0.01	+0.2
	sulfamerazine	6.91	6.53	–0.38	–5.5
	sulfamethoxazole	7.80	7.49	–0.31	–3.9
	sulfamethazine	8.94	8.64	–0.30	–3.4

As pointed out before, the method shown in Figure 3-4 a has a start temperature of the gradient of 60 °C. In order to shorten the analysis time and concurrently increase sample throughput it is possible to choose a higher start temperature than 60 °C for the temperature gradient. In this case, new k_0 values of all analytes at the desired start temperature are required which can be calculated from the $\ln k$ vs T plot. Hence, the operator should perform two additional isothermal/isocratic runs. To verify this approach a temperature of 100 °C was chosen as the new start temperature of the temperature gradient and isothermal/isocratic retention data from 60 °C and 180 °C were employed to build the $\ln k$ vs T plot. Afterwards, a linear regression of this plot was performed to calculate the required k_0 values for each sulfonamide which correspond to a temperature of 100 °C. The upper temperature limit was again set to 180 °C. The required S_T values were taken from the fitting of the temperature-gradient runs in the temperature range from 60 °C to 180 °C. Subsequently, a method for the separation of the sulfonamides was developed and is shown in Figure 3-4 b.

A baseline separation of the analytes can be achieved within approximately 9.5 minutes with a critical resolution between sulfadiazine and sulfathiazole of 2.1. Moreover, the method consists of three segments, two isothermal holds at 100 and 180 °C and a linear temperature gradient with a slope of 25 °C min^{-1}. The isothermal hold at the beginning of the chromatogram was necessary to avoid co-elution of sulfadiazine and sulfathiazole. As can be seen from Table 3-4 predicted retention times are also in good agreement with the experimental data except for sulfamerazine where a relative error of 5.5% was observed. For

the other analytes, a relative error less than 4% and an average relative error of 2.9% were calculated. From a practical point of view, the method shown in Figure 3-4 a might be preferred using special hyphenation techniques because of the higher resolution between the critical peak pair. Moreover, for method comparison the column lifetime, especially at temperatures higher than 150 °C, has also to be considered. For both methods shown in Figure 3-4, the column is exposed to a temperature higher than 150 °C for approximately 4 minutes, but the methods differ in the isothermal hold at 180 °C. The temperature gradient shown in Figure 3-4 a contains an isothermal hold at 180 °C for 1 minute, whereas the column is exposed for 3 minutes to 180 °C using the method shown in Figure 3-4 b. In order to increase the column lifetime, the separation method shown in Figure 3-4 a will be preferred.

Considering the accuracy of temperature-gradient predictions under isocratic conditions in LC, it can be pointed out that the results shown in this study are comparable to the results of Nikitas and Pappa-Louisi. For example, Pappa-Louisi et al. [27] reported an average relative error of 2.4% for predicted retention times within a temperature interval from 15 °C up to 75 °C (ΔT = 60 °C) where an isocratic mobile phase (40% acetonitrile) was employed. During their work linear temperature gradients with slopes of 2.0, 3.0, 4.0 and 10.0 °C min^{-1} were investigated using a conventional-bore HPLC column (150 × 4.6 mm, 3.5 µm). In our study, a twofold larger temperature interval from 60 °C up to 180 °C (ΔT = 120 °C) was investigated. Moreover, within this range complex segmented temperature gradients with slopes of 12 °C min^{-1} (Figure 3-4 a) and 25 °C min^{-1} (Figure 3-4 b) were predicted.

3.4.4 Comparison of the Isothermal and Temperature-Gradient Method

In this section, the developed isothermal as well as temperature-gradient method of the sulfonamide separations will be compared. The corresponding chromatograms of both methods are depicted in Figure 3-5.

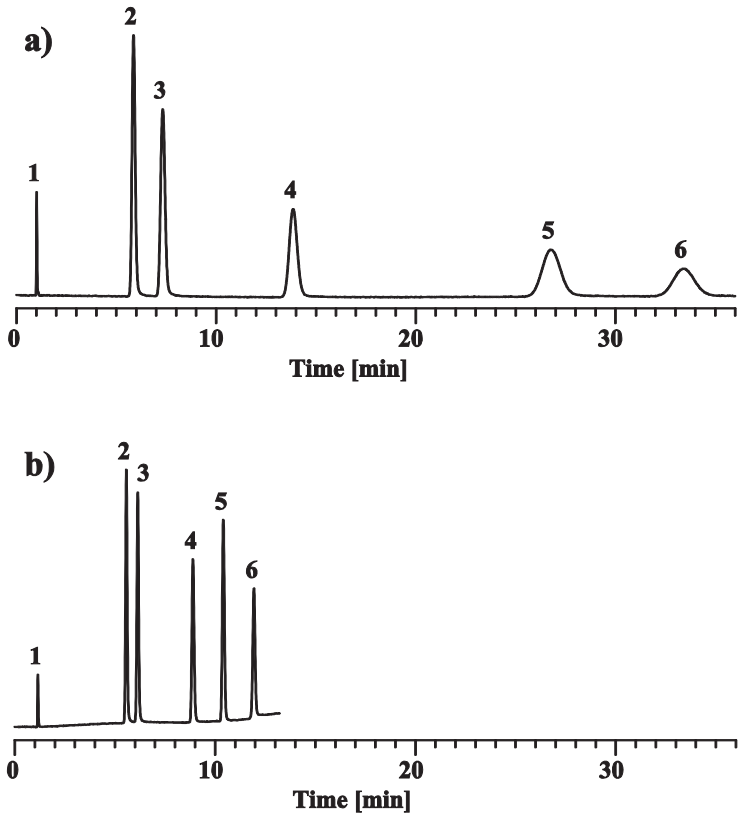

Figure 3-5: Comparison between the a) isothermal and b) temperature-gradient elution of selected sulfonamides. Chromatographic conditions: stationary phase: Waters XBridge C_{18} (75 × 4.6 mm, 2.5 µm); mobile phase: deionized water with 0.1% formic acid; flow rate: 1.0 mL min^{-1}; temperature: a) 80 °C, b) temperature gradient the same as in Figure 3-4 a; injection volume: a) 2 µL, b) 1 µL. Analytes: 1) uracil, 2) sulfadiazine, 3) sulfathiazole, 4) sulfamerazine, 5) sulfamethoxazole, 6) sulfamethazine.

As can be seen, the analysis time of the developed temperature gradient method shown in Figure 3-5 b is three times faster than the isothermal separation at 80 °C (Figure 3-5 a). More practically relevant than the analysis time will be the total analysis time including the cycle time which is required to cool down the system. In this study the cycle time is referred to a state of full equilibration as opposed to a dynamic equilibration. This means that the retention time of the solutes are independent of the re-equilibration time [57]. For the column oven in this study, a cycle time of 8 minutes was determined to cool down the system from 180 °C to

60 °C (data not shown here). Therefore, a total analysis time of the developed temperature-gradient method of 21 minutes was calculated. Considering the total analysis time, the temperature-gradient method is 1.7-times faster than the isothermal method at 80 °C. Furthermore, in temperature-programming mode, narrower and higher peaks can be obtained which result in lower limits of detection. In our opinion, temperature-programming is the method of choice for the separation of the selected sulfonamides, although column lifetime can be prolonged when working at 80 °C.

3.5 Conclusion

In this study it was investigated whether isothermal retention times can be predicted based on two temperature-gradient input measurements by means of equation 3-7. It was shown that these predictions failed and a major relative error of predicted isothermal retention factors up to 169.6% was observed. However, such isothermal retention factor predictions are required to predict temperature gradients with different start temperatures. To overcome this issue, additional isothermal data have been employed. In this context it was shown that isothermal predictions based on a linear regression of a plot of $\ln k$ vs T results in more reliable predictions than a plot of $\ln k$ vs $1/T$. Therefore, a plot of $\ln k$ vs T has been employed to calculate the required retention factors of the analytes corresponding to the selected start temperature. This means that, if a change of the start temperature of the predicted temperature gradient is necessary during method development, at least four experimental measurements are required, two temperature gradient and two isothermal runs. Retention times for segmented temperature gradients could then be predicted with an average relative error of 2.9%.

In contrast, in the case where a change of the start temperature of the predicted temperature gradient is not necessary, only two basic measurements are required to predict segmented temperature gradients with an average relative error of 2.2%.

The comparison of the observed relative errors using both approaches indicates that the LES parameter S_T should be calculated temperature dependent in order to improve the reliability of LES based predictions even further. Moreover, it would also be advantageous to extend the LES model in order to predict temperature and flow rate gradients simultaneously in order to operate the column in its van Deemter minimum as well as to shorten the analysis time even more.

Furthermore, the application of the described temperature-programming approach is not restricted to very polar analytes like sulfonamides. Temperature-programming method development can also be applied to non-polar analytes such as steroids [29,51]. In this case, a column has to be chosen which is less hydrophobic than hybrid silica based C_{18} columns, because the elution strength of a water mobile phase is too low, even at temperatures as high as 200 °C. For this reason, metal oxide-based columns such as polymer coated zirconium dioxide would be suitable.

Acknowledgment

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3.6 References

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Appendix for Chapter 3

Abstract

The Appendix for Chapter 3 contains additional information about how MS Excel Solver can be employed to calculate the required model parameters as well as how the retention time of an analyte can be calculated depending on a segmented-temperature gradient in liquid chromatography.

Contents

Eight Figures and two Tables.

Appendix 3-1. Determination of the Model Parameters S_T and k_0

For the determination of the parameters S_T and k_0 of the LES model equations A 3-1 and A 3-2 are required.

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

$$b_T = \frac{t_0 S_T \Delta T}{tG} \quad \text{(A 3-2)}$$

The combination of both equations yields

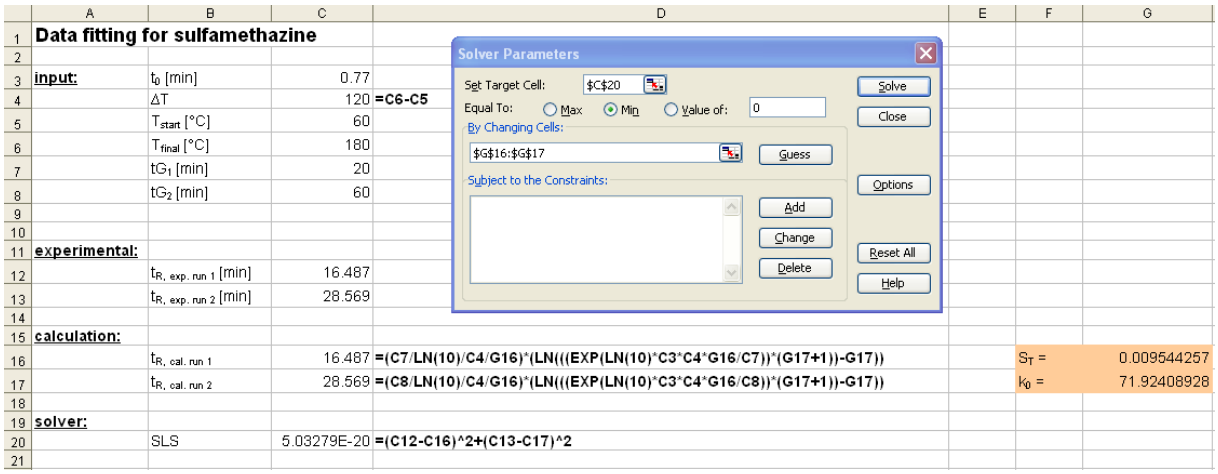
$$t_R = \frac{tG}{2.3 \Delta T S_T} \ln\left[e^{\frac{2.3 t_0 \Delta T S_T}{tG}} \left(k_0 + 1\right) - k_0 \right] \quad \text{(A 3-3)}$$

Using equation A 3-3, at least two temperature-gradient input measurements are required. These runs should differ in the gradient slope of a factor of three. Moreover, selected analytes should elute during the applied temperature gradient. Table A 3-1 contains experimentally obtained retention times of selected sulfonamides for two temperature-gradient measurements from 60 °C to 180 °C within 20 minutes (6 °C min^{-1}) and 60 minutes (2 °C min^{-1}).

Table A 3-1: Overview of experimentally obtained retention times of selected sulfonamides of the basis input measurements. Temperature interval 60 °C - 180 °C.

analyte	**experimental retention time [min], 2 °C min^{-1}**	**experimental retention time [min], 6 °C min^{-1}**
uracil	1.160	1.156
sulfadiazine	8.340	6.978
sulfathiazole	10.660	8.071
sulfamerazine	16.671	11.579
sulfamethoxazole	24.547	14.215
sulfamethazine	28.569	16.487

The numerical solution of equation A 3-3 can be achieved using a spreadsheet calculator such as Microsoft Excel Solver. In Figure A 3-1 a screenshot of the spreadsheet calculator is presented.



	A	B	C	D	E	F	G
1	**Data fitting for sulfamethazine**						
2							
3	**input:**	t_0 [min]	0.77				
4		ΔT	120	=C6-C5			
5		T_{start} [°C]	60				
6		T_{final} [°C]	180				
7		tG_1 [min]	20				
8		tG_2 [min]	60				
9							
10							
11	**experimental:**						
12		$t_{R,\ exp.\ run\ 1}$ [min]	16.487				
13		$t_{R,\ exp.\ run\ 2}$ [min]	28.569				
14							
15	**calculation:**						
16		$t_{R,\ cal.\ run\ 1}$	16.487	=(C7/LN(10)/C4/G16)*(LN(((EXP(LN(10)*C3*C4*G16/C7))*(G17+1))-G17))		S_T =	0.009544257
17		$t_{R,\ cal.\ run\ 2}$	28.569	=(C8/LN(10)/C4/G16)*(LN(((EXP(LN(10)*C3*C4*G16/C8))*(G17+1))-G17))		k_0 =	71.92408928
18							
19	**solver:**						
20		SLS	5.03279E-20	=(C12-C16)^2+(C13-C17)^2			
21							

Figure A 3-1: Spreadsheet calculator, sheet displaying the equations that are needed to calculate S_T and k_0.

Input:

A number of experimental data have to be determined and specified.

- the column dead time (t_0)
- the start temperature of the temperature gradient (T_{start})
- the final temperature of the temperature gradient (T_{final})
- the change in temperature during the temperature gradient ($\Delta T = T_{final} - T_{start}$)
- the gradient time of the first temperature gradient (tG_1)
- the gradient time of the second temperature gradient (tG_2)

Moreover, two additional cells have to be defined for S_T and k_0.

Experimental:

The experimentally obtained retention times of the selected analyte (sulfamethazine) for the first and the second temperature gradient have to be specified in the spreadsheet calculator.

- experimentally obtained retention time for the first gradient ($t_{R,\exp.run1}$[min])
- experimentally obtained retention time for the second gradient ($t_{R,\exp.run2}$[min])

Calculation:

Here, two cells have to be specified where the theoretical retention times of the selected analyte (sulfamethazine) are calculated according to equation A 3-3 for the first and second temperature gradient.

- theoretical retention for the first gradient ($t_{R,cal.run1}$ [min])
- theoretical retention for the first gradient ($t_{R,cal.run2}$ [min])

Solver:

Furthermore, a cell is implemented where the sum of the least squares (SLS) is calculated, representing the differences between calculated and experimental retention times for the first and second temperature gradient.

$$SLS = \left(t_{R,cal.\,run1} - t_{R,\exp.\,run1}\right)^2 + \left(t_{R,cal.\,run2} - t_{R,\exp.\,run2}\right)^2$$

Microsoft Excel Solver is then employed to calculate the values of S_T and k_0 for the selected analyte depending on the sum of the least squares being as small as possible. The "Target Cell" is the cell which corresponds to the SLS and should be set "Equal To: Min" or a "Value of" 0. The cells which are corresponding to S_T and k_0 are the cells used by MS Excel Solver to solve the system of equations. Following, the Solver function will search for values of S_T and k_0 according to equation A 3-3 yielding the same retention times as those obtained experimentally.

The described procedure has to be performed for each analyte in the sample mixture.

Appendix 3-2. Calculation of the Retention Time of Sulfamethazine for the Separation shown in Figure 3-4 a

Appendix 3-2.1. Required Equations

To calculate the retention time of an analyte for a segmented temperature gradient, equations A 3-4 to A 3-6 are needed. Equation A 3-4 describes the retention time of an analyte during a certain temperature segment. Equation A 3-5 can be employed to calculate the change of the retention factor of an analyte depending on the applied temperature gradient of a temperature segment. This value of the retention factor represents the start value of k_0 for the next temperature segment. In order to calculate the retention time of an analyte for an isothermal/isocratic hold equation A 3-6 should be employed.

$$t_R = \frac{t_0}{2.3 b_T} \ln\left[e^{2.3 b_T r} \left(k_0 + 1\right) - k_0 \right] \quad \text{(A 3-4)}$$

$$\log k_r = \log k_0 - \frac{b_T t_R}{t_0} \quad \text{(A 3-5)}$$

$$t_R = r t_0 \left(k_0 + 1\right) \quad \text{(A 3-6)}$$

Appendix 3-2.2. Calculation of the Retention Time of Sulfamethazine for the First Temperature Segment of the Separation shown in Figure 3-4 a

First, a number of experimental/theoretical data has to be determined and specified (Figure A 3-2).

- the column dead time t_0 (cell: G11)
- the start temperature of the temperature gradient T_{start} (cell: G12)
- the final temperature of the temperature gradient T_{final} (cell: G13)
- the change in temperature during the temperature segment (cell: G15)
- the gradient time of the temperature segment tG_1 (cell: G16)
- a cell where the temperature-gradient steepness parameter b_T will be calculated according to equation A 3-2 (cell: C12)

- a cell for the fractional migration $r_{segment1}$ of the analyte during the first temperature segment (cell: C13)
- two cells containing the values of S_T (cell: C5) and k_0 (cell: C6) which were calculated using the approach described in section Appendix 3-1
- a cell where the retention time will be calculated according to equation A 3-4 (cell: C15)
- a cell where the change of the retention factors during the first segment will be calculated according to equation A 3-5 (cells C16 and C17)

Figure A 3-2 shows a screenshot of the spreadsheet calculator which has been employed to calculate the retention time of sulfamethazine for the first temperature segment.

	A	B	C	D	E	F	G
1		**Calculation of the retention time of sulfamethazine of figure 4a**					
2						applied temperature gradient	
3							
4		**parameter**	**sulfamethazine**			time	temp. [°C]
5		S_T	0.009544257	=Data_fitting_sulfamethazine!G16		0.00	60
6		k_0	71.92408928	=Data_fitting_sulfamethazine!G17		4.41	115
7		column length [mm]	75			5.99	115
8						11.61	180
9						12.50	180
10							
11		**segment 1**				t_0	0.770
12		$b_{T\ segment\ 1}$	0.091655163	=$G$11*$G$15*$C$5/$G$16		T_{start}	60
13		$r_{segment\ 1}$	1			T_{final}	115
14		fractional migration segment 1 [mm]	75	=$C$7*C13			
15		$t_{R\ segment\ 1}$	**10.57281702**	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	55
16		log $k_{f\ segment\ 1}$	0.598363633	=LOG10($C$6)-($C$12*$C15/$G$11)		$tG_{segment\ 1}$	4.41
17		$k_{f\ segment\ 1}$	3.966099754	=10^C16		slope [°C/min]	12.5
19		**segment 2 (isothermal/isocratic)**				t_0	0.770
20		$b_{T\ segment\ 2}$	0	=$G$19*$G$23*$C$5/$G$24		T_{start}	115
21		$r_{segment\ 2}$	0			T_{final}	115
22		fractional migration segment 2 [mm]	0	=$C$7*C21			
23		$t_{R\ segment\ 2}$	**0.00**	=$G$19*$C$21*($C$17+1)		$\Delta T_{segment\ 2}$	0
24		log $k_{f\ segment\ 2}$	0.598363633	=C16		$tG_{segment\ 2}$	1.58
25		$k_{f\ segment\ 2}$	3.966099754	=C17		slope [°C/min]	0.0
27		**segment 3**				t_0	0.770
28		$b_{T\ segment\ 3}$	0.084998229	=($G$27*$G$31*$C$5/$G$32)		T_{start}	115
29		$r_{segment\ 3}$	0			T_{final}	180
30		fractional migration segment 3 [mm]	0	=$C$7*C29			
31		$t_{R\ segment\ 3}$	**0**	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25))		$\Delta T_{segment\ 3}$	65
32		log $k_{f\ segment\ 3}$	0.598363633	=LOG10($C$6)-($C$12*$C15/$G$11)-($C$28*$C31/$G$27)		$tG_{segment\ 3}$	5.62
33		$k_{f\ segment\ 3}$	3.966099754	=10^C32		slope [°C/min]	11.6
35		**segment 4 (isothermal/isocratic)**				t_0	0.770
36		$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)		T_{start}	180
37		$r_{segment\ 4}$	0	=(1-C13-C21-C29)		T_{final}	180
38		fractional migration segment 1 [mm]	0	=$C$7*C37			
39		$t_{R\ segment\ 4}$	**0**	=$G$35*$C$37*($C$33+1)		$\Delta T_{segment\ 4}$	0
40		log $k_{f\ segment\ 4}$	0.598363633	=C32		$tG_{segment\ 4}$	1
41		$k_{f\ segment\ 4}$	3.966099754	=10^C40		slope [°C/min]	0.0

Data_fitting_sulfamethazine | Data_fitting_total | **Figure_4a_sulfamethazine** | Figure_4a_total | calculation k0 at 100 °C | Figure_4b_sulfamethazine | Figure_4b_total | calcl

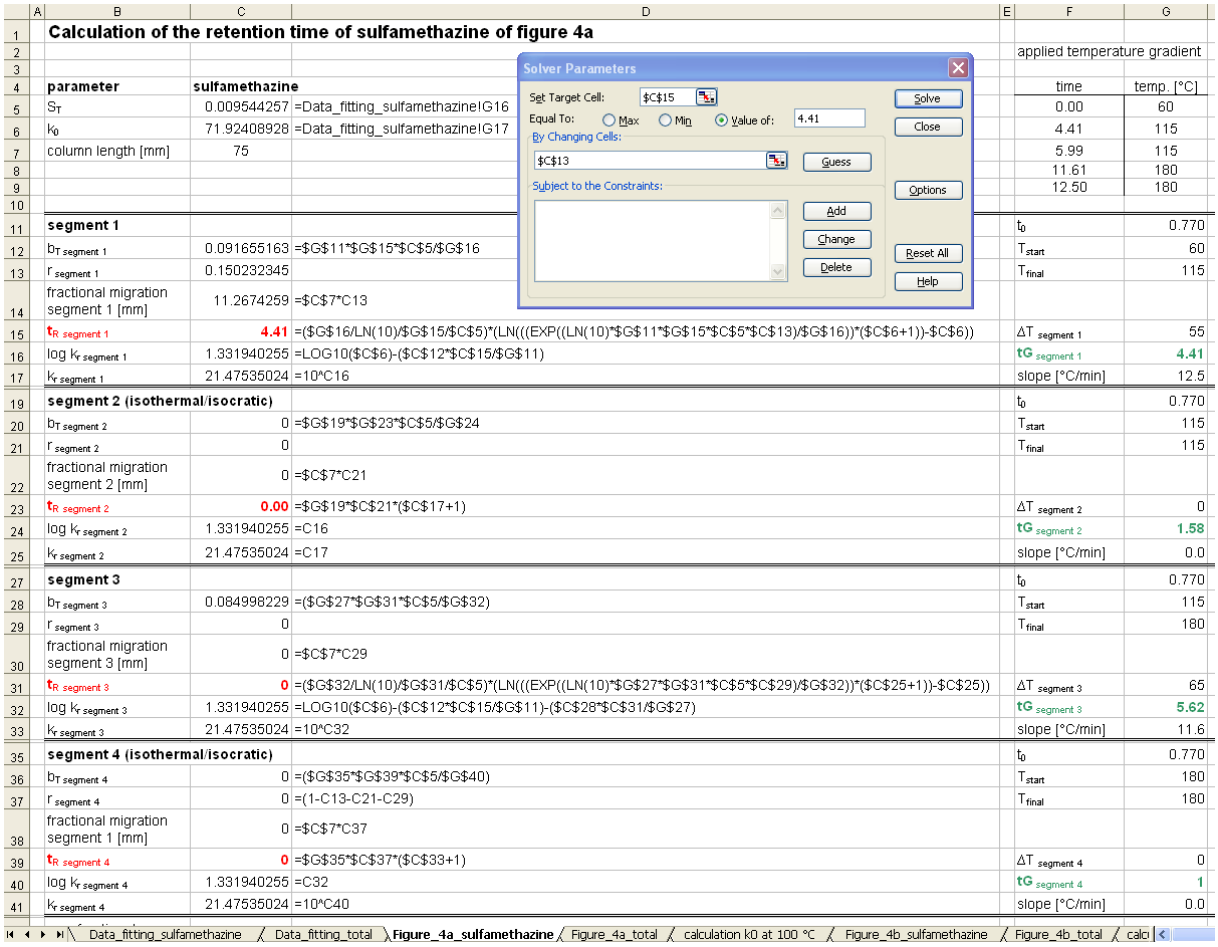
Figure A 3-2: Spreadsheet calculator, sheet displaying the equations that are needed to calculate the retention time and fractional migration of sulfamethazine for the first temperature segment.

The calculation starts by setting the value of the fractional migration of sulfamethazine for the first temperature segment to 1, assuming that the analyte migrates completely during the first segment. As can be seen from Figure A 3-2, this setting yields a retention time of sulfamethazine of 10.57 min (cell: C15). It has to be considered that the maximal retention time of sulfamethazine for the first segment can only be equal or lower to the temperature-gradient time of the first segment (cell: G16). Hence, the maximal retention time of sulfamethazine can only be 4.41 min or lower. In other words, if the calculated retention time of an analyte for a temperature segment is higher than the temperature-gradient time of the segment, the analyte does not elute during this temperature segment. This means that the analyte only migrates a certain distance of the total column length during the temperature segment.

Therefore, the fractional migration of the analyte has to be calculated depending on the applied temperature gradient of the first temperature segment. Figure A 3-3 shows a screenshot of the spreadsheet calculator which has been employed to calculate the fractional migration of sulfamethazine for the first temperature segment by means of MS Excel Solver.

The “Target Cell” corresponds to the retention time of sulfamethazine for the first temperature segment (cell: C15) and should be set “Equal To” a “Value of” 4.41 “By Changing Cells” that corresponds with the fractional migration (cell: C13) of sulfamethazine during the first temperature segment. Now, the solver function will search for a value of the fractional migration ($r_{segment1}$), yielding a retention time of sulfamethazine of 4.41 min. As can be seen from Figure A 3-3, the retention time of sulfamethazine was calculated to 4.41 min for the first temperature segment by changing the fractional migration to a value of approximately 0.1502 corresponding to a migration distance of sulfamethazine of 11.27 mm of the total column length during this temperature segment.

As pointed out before, because of the applied temperature gradient during the first segment, the value of the retention factor (k_0) of sulfamethazine was changed. Now, the retention factor of sulfamethazine corresponding with the final temperature of the first segment which is equal to the start temperature of the second temperature segment has to be calculated according to equation A 3-5 and yields a value of 21.48 (cell: C17).



Calculation of the retention time of sulfamethazine of figure 4a

parameter	sulfamethazine	
S_T	0.009544257	=Data_fitting_sulfamethazine!G16
k_0	71.92408928	=Data_fitting_sulfamethazine!G17
column length [mm]	75	
segment 1		
$b_{T\ segment\ 1}$	0.091655163	=$G$11*$G$15*$C$5/$G$16
$r_{segment\ 1}$	0.150232345	
fractional migration segment 1 [mm]	11.2674259	=$C$7*C13
$t_{R\ segment\ 1}$	4.41	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))
log $k_{F\ segment\ 1}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)
$k_{F\ segment\ 1}$	21.47535024	=10^C16
segment 2 (isothermal/isocratic)		
$b_{T\ segment\ 2}$	0	=$G$19*$G$23*$C$5/$G$24
$r_{segment\ 2}$	0	
fractional migration segment 2 [mm]	0	=$C$7*C21
$t_{R\ segment\ 2}$	0.00	=$G$19*$C$21*($C$17+1)
log $k_{F\ segment\ 2}$	1.331940255	=C16
$k_{F\ segment\ 2}$	21.47535024	=C17
segment 3		
$b_{T\ segment\ 3}$	0.084998229	=($G$27*$G$31*$C$5/$G$32)
$r_{segment\ 3}$	0	
fractional migration segment 3 [mm]	0	=$C$7*C29
$t_{R\ segment\ 3}$	0	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25))
log $k_{F\ segment\ 3}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)-($C$28*$C$31/$G$27)
$k_{F\ segment\ 3}$	21.47535024	=10^C32
segment 4 (isothermal/isocratic)		
$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)
$r_{segment\ 4}$	0	=(1-C13-C21-C29)
fractional migration segment 1 [mm]	0	=$C$7*C37
$t_{R\ segment\ 4}$	0	=$G$35*$C$37*($C$33+1)
log $k_{F\ segment\ 4}$	1.331940255	=C32
$k_{F\ segment\ 4}$	21.47535024	=10^C40

applied temperature gradient

time	temp. [°C]
0.00	60
4.41	115
5.99	115
11.61	180
12.50	180

t_0	0.770	t_0	0.770
T_{start}	60	T_{start}	115
T_{final}	115	T_{final}	115
$\Delta T_{segment\ 1}$	55	$\Delta T_{segment\ 2}$	0
$tG_{segment\ 1}$	4.41	$tG_{segment\ 2}$	1.58
slope [°C/min]	12.5	slope [°C/min]	0.0

t_0	0.770	t_0	0.770
T_{start}	115	T_{start}	180
T_{final}	180	T_{final}	180
$\Delta T_{segment\ 3}$	65	$\Delta T_{segment\ 4}$	0
$tG_{segment\ 3}$	5.62	$tG_{segment\ 4}$	1
slope [°C/min]	11.6	slope [°C/min]	0.0

Solver Parameters: Set Target Cell: $C$15; Equal To: Max / Min / Value of: 4.41; By Changing Cells: $C$13; Subject to the Constraints:; Solve, Close, Guess, Options, Add, Change, Delete, Reset All, Help

Data_fitting_sulfamethazine | Data_fitting_total | Figure_4a_sulfamethazine | Figure_4a_total | calculation k0 at 100 °C | Figure_4b_sulfamethazine | Figure_4b_total | calci

Figure A 3-3: Spreadsheet calculator, sheet displaying the equations and Solver Parameters that are needed to calculate the fractional migration of sulfamethazine for the first temperature segment.

Appendix 3-2.3. Calculation of the Retention Time of Sulfamethazine for the Second Temperature Segment of the Separation shown in Figure 3-4 a

It has to be considered that the second segment of the temperature-gradient method is an isothermal/isocratic hold. Therefore, the retention time of sulfamethazine has to be calculated according to equation A 3-6 instead of equation A 3-4.

Very similar to the calculation of the retention time of sulfamethazine for the first temperature segment, a number of experimental/theoretical data have to be determined and specified for the second segment (Figure A 3-4).

- the column dead time t_0 (cell: G19)
- the start temperature of the temperature gradient T_{start} (cell: G20)
- the final temperature of the temperature gradient T_{final} (cell: G21)
- the change in temperature during the temperature segment (cell: G23)
- the gradient time of the temperature segment tG_2 (cell: G24)
- a cell where the temperature-gradient steepness parameter b_T will be calculated according to equation A 3-2 (cell: C20)
- a cell for the fractional migration $r_{segment\,2}$ of the analyte during the second temperature segment (cell: C21)
- a cell containing the value of S_T (cell: C5)
- a cell containing the value of k_0 corresponding to the value of the retention factor calculated after the first temperature segment (cell: C17)
- a cell where the retention time will be calculated according to equation A 3-6 (cell: C23)
- a cell where the change of the retention factor during the second segment will be calculated according to equation A 3-5 (cells C24 and C25)

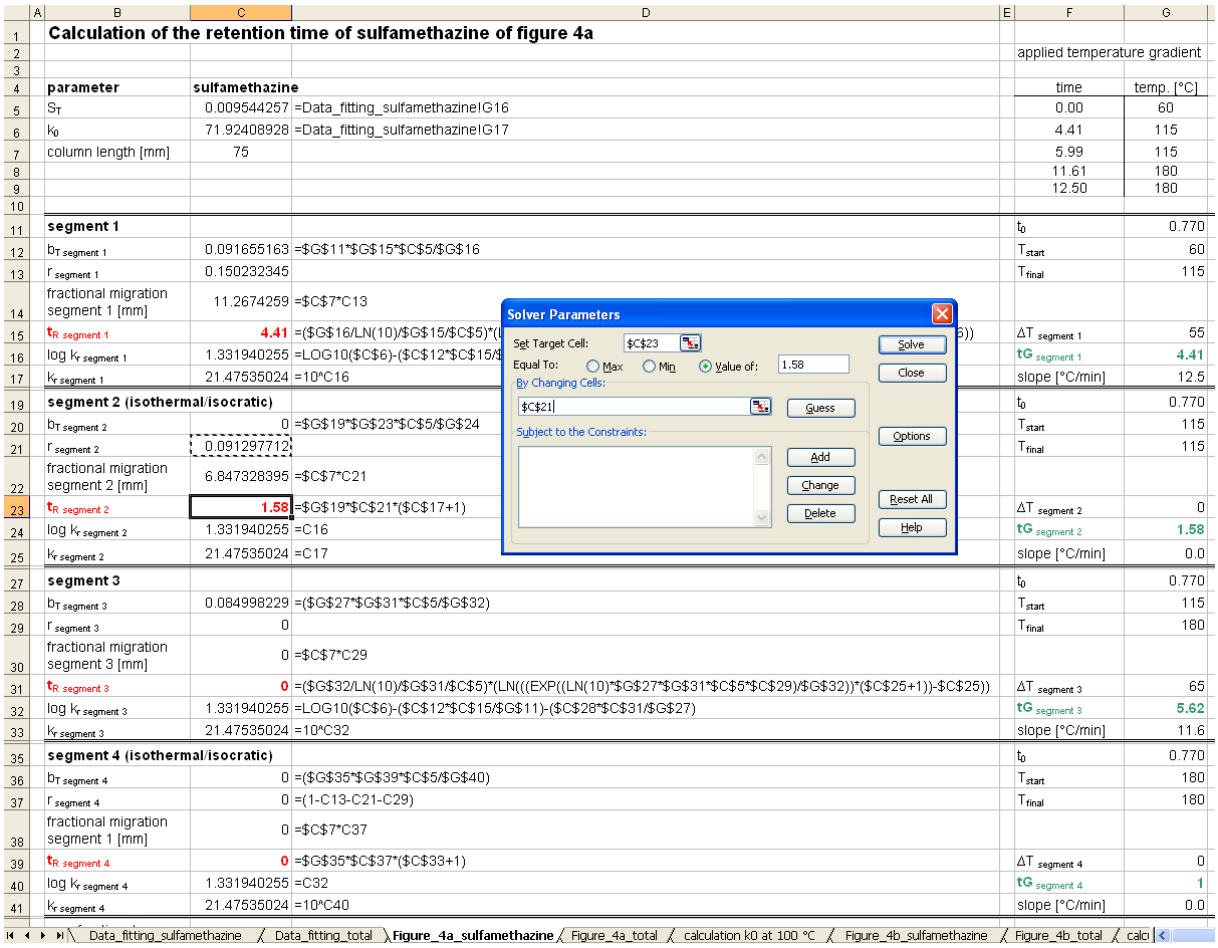
Figure A 3-4 shows a screenshot of the spreadsheet calculator which has been employed to calculate the retention time of sulfamethazine for the second temperature segment.

	A	B	C	D	E	F	G
1		**Calculation of the retention time of sulfamethazine of figure 4a**					
2						applied temperature gradient	
3							
4		**parameter**	**sulfamethazine**			time	temp. [°C]
5		S_T	0.009544257	=Data_fitting_sulfamethazine!G16		0.00	60
6		k_0	71.92408928	=Data_fitting_sulfamethazine!G17		4.41	115
7		column length [mm]	75			5.99	115
8						11.61	180
9						12.50	180
10							
11		**segment 1**				t_0	0.770
12		$b_{T\ segment\ 1}$	0.091655163	=$G$11*$G$15*$C$5/$G$16		T_{start}	60
13		$r_{segment\ 1}$	0.150232345			T_{final}	115
14		fractional migration segment 1 [mm]	11.2674259	=$C$7*C13			
15		$t_{R\ segment\ 1}$	**4.41**	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	55
16		log $k_{F\ segment\ 1}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)		$tG_{segment\ 1}$	**4.41**
17		$k_{F\ segment\ 1}$	21.47535024	=10^C16		slope [°C/min]	12.5
19		**segment 2 (isothermal/isocratic)**				t_0	0.770
20		$b_{T\ segment\ 2}$	0	=$G$19*$G$23*$C$5/$G$24		T_{start}	115
21		$r_{segment\ 2}$	0.849767655			T_{final}	115
22		fractional migration segment 2 [mm]	63.7325741	=$C$7*C21			
23		$t_{R\ segment\ 2}$	**14.71**	=$G$19*$C$21*($C$17+1)		$\Delta T_{segment\ 2}$	0
24		log $k_{F\ segment\ 2}$	1.331940255	=C16		$tG_{segment\ 2}$	**1.58**
25		$k_{F\ segment\ 2}$	21.47535024	=C17		slope [°C/min]	0.0
27		**segment 3**				t_0	0.770
28		$b_{T\ segment\ 3}$	0.084998229	=($G$27*$G$31*$C$5/$G$32)		T_{start}	115
29		$r_{segment\ 3}$	0			T_{final}	180
30		fractional migration segment 3 [mm]	0	=$C$7*C29			
31		$t_{R\ segment\ 3}$	**0**	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25))		$\Delta T_{segment\ 3}$	65
32		log $k_{F\ segment\ 3}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)-($C$28*$C$31/$G$27)		$tG_{segment\ 3}$	**5.62**
33		$k_{F\ segment\ 3}$	21.47535024	=10^C32		slope [°C/min]	11.6
35		**segment 4 (isothermal/isocratic)**				t_0	0.770
36		$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)		T_{start}	180
37		$r_{segment\ 4}$	0	=(1-C13-C21-C29)		T_{final}	180
38		fractional migration segment 1 [mm]	0	=$C$7*C37			
39		$t_{R\ segment\ 4}$	**0**	=$G$35*$C$37*($C$33+1)		$\Delta T_{segment\ 4}$	0
40		log $k_{F\ segment\ 4}$	1.331940255	=C32		$tG_{segment\ 4}$	**1**
41		$k_{F\ segment\ 4}$	21.47535024	=10^C40		slope [°C/min]	0.0

Data_fitting_sulfamethazine | Data_fitting_total | **Figure_4a_sulfamethazine** | Figure_4a_total | calculation k0 at 100 °C | Figure_4b_sulfamethazine | Figure_4b_total | calci

Figure A 3-4: Spreadsheet calculator, sheet displaying the equations that are needed to calculate the retention time and fractional migration of sulfamethazine for the second temperature segment.

The calculation starts with the assumption that sulfamethazine will elute completely during the second segment. Therefore, the value for the fractional migration $r_{segment\,2}$ of sulfamethazine will be set to 0.849 ($1-r_{segment\,1}=0.849$). Doing so, a retention time of sulfamethazine of 14.71 min was calculated (cell: C23). It has to be considered that the temperature-gradient time ($tG_{segment\,2}$) of the second segment was set to 1.58 min. Therefore, MS Excel Solver has to be employed to find a value for $r_{segment\,2}$ yielding a retention time for sulfamethazine of 1.58 min for the second temperature segment. Figure A 3-5 depicts the necessary equations and Solver Parameters.



	B	C	D	F	G
1	**Calculation of the retention time of sulfamethazine of figure 4a**				
2				applied temperature gradient	
3					
4	**parameter**	**sulfamethazine**		time	temp. [°C]
5	S_T	0.009544257	=Data_fitting_sulfamethazine!G16	0.00	60
6	k_0	71.92408928	=Data_fitting_sulfamethazine!G17	4.41	115
7	column length [mm]	75		5.99	115
8				11.61	180
9				12.50	180
10					
11	**segment 1**			t_0	0.770
12	$b_{T\ segment\ 1}$	0.091655163	=$G$11*$G$15*$C$5/$G$16	T_{start}	60
13	$r_{segment\ 1}$	0.150232345		T_{final}	115
14	fractional migration segment 1 [mm]	11.2674259	=$C$7*C13		
15	$t_{R\ segment\ 1}$	4.41	=($G$16/LN(10)/$G$15/$C$5)*(I	$\Delta T_{segment\ 1}$	55
16	log $k_{f\ segment\ 1}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$	$tG_{segment\ 1}$	4.41
17	$k_{f\ segment\ 1}$	21.47535024	=10^C16	slope [°C/min]	12.5
19	**segment 2 (isothermal/isocratic)**			t_0	0.770
20	$b_{T\ segment\ 2}$	0	=$G$19*$G$23*$C$5/$G$24	T_{start}	115
21	$r_{segment\ 2}$	0.091297712		T_{final}	115
22	fractional migration segment 2 [mm]	6.847328395	=$C$7*C21		
23	$t_{R\ segment\ 2}$	1.58	=$G$19*$C$21*($C$17+1)	$\Delta T_{segment\ 2}$	0
24	log $k_{f\ segment\ 2}$	1.331940255	=C16	$tG_{segment\ 2}$	1.58
25	$k_{f\ segment\ 2}$	21.47535024	=C17	slope [°C/min]	0.0
27	**segment 3**			t_0	0.770
28	$b_{T\ segment\ 3}$	0.084998229	=($G$27*$G$31*$C$5/$G$32)	T_{start}	115
29	$r_{segment\ 3}$	0		T_{final}	180
30	fractional migration segment 3 [mm]	0	=$C$7*C29		
31	$t_{R\ segment\ 3}$	0	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25))	$\Delta T_{segment\ 3}$	65
32	log $k_{f\ segment\ 3}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)-($C$28*$C$31/$G$27)	$tG_{segment\ 3}$	5.62
33	$k_{f\ segment\ 3}$	21.47535024	=10^C32	slope [°C/min]	11.6
35	**segment 4 (isothermal/isocratic)**			t_0	0.770
36	$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)	T_{start}	180
37	$r_{segment\ 4}$	0	=(1-C13-C21-C29)	T_{final}	180
38	fractional migration segment 1 [mm]	0	=$C$7*C37		
39	$t_{R\ segment\ 4}$	0	=$G$35*$C$37*($C$33+1)	$\Delta T_{segment\ 4}$	0
40	log $k_{f\ segment\ 4}$	1.331940255	=C32	$tG_{segment\ 4}$	1
41	$k_{f\ segment\ 4}$	21.47535024	=10^C40	slope [°C/min]	0.0

Figure A 3-5: Spreadsheet calculator, sheet displaying the equations and Solver Parameters that are needed to calculate the fractional migration of sulfamethazine for the second temperature segment.

The "Target Cell" corresponds to the retention time of sulfamethazine for the second temperature segment (cell: C23) and should be set "Equal To" a "Value of" 1.58 "By Changing Cells" that corresponds to the fractional migration (cell: C21) of sulfamethazine during the second segment. Afterwards, the solver function will search for a value of $r_{segment\,2}$ yielding a retention time of 1.58 min for sulfamethazine. A value of approximately 0.0913 gives the desired retention time and corresponds to a migration distance of sulfamethazine of 6.85 mm of the total column length during the second temperature segment.

The second temperature segment of the method is an isothermal/isocratic hold, hence the value of the retention factor after this segment equals the value at the start of the second temperature segment.

Appendix 3-2.4. Calculation of the Retention Time of Sulfamethazine for the Third Temperature Segment of the Separation shown in Figure 3-4 a

For the calculation of the retention of sulfamethazine for the third temperature segment, a number of experimental/theoretical data has to be determined and specified (Figure A 3-6).

- the column dead time t_0 (cell: G27)
- the start temperature of the temperature gradient T_{start} (cell: G28)
- the final temperature of the temperature gradient T_{final} (cell: G29)
- the change in temperature during the temperature segment (cell: G31)
- the gradient time of the temperature segment tG_3 (cell: G32)
- a cell where the temperature-gradient steepness parameter b_T will be calculated according to equation A 3-2 (cell: C28)
- a cell for the fractional migration $r_{segment\,3}$ of the analyte during the third temperature segment (cell: C29)
- a cell containing the value of S_T (cell: C5)
- a cell containing the value of k_0 corresponding to the value of the retention factor calculated after the second temperature segment (cell: C25)
- a cell where the retention time will be calculated according to equation A 3-4 (cell: C31)
- a cell where the change of the retention factor during the third segment will be calculated according to equation A 3-5 (cells C32 and C33)

Figure A 3-6 depicts a screenshot of the spreadsheet calculator which has been employed to calculate the retention time of sulfamethazine for the third temperature segment.

	A	B	C	D	E	F	G
1		**Calculation of the retention time of sulfamethazine of figure 4a**					
2						applied temperature gradient	
3							
4		**parameter**	**sulfamethazine**			time	temp. [°C]
5		S_T	0.009544257	=Data_fitting_sulfamethazine!G16		0.00	60
6		k_0	71.92408928	=Data_fitting_sulfamethazine!G17		4.41	115
7		column length [mm]	75			5.99	115
8						11.61	180
9						12.50	180
10							
11		**segment 1**				t_0	0.770
12		$b_{T\ segment\ 1}$	0.091655163	=$G$11*$G$15*$C$5/$G$16		T_{start}	60
13		$r_{segment\ 1}$	0.150232345			T_{final}	115
14		fractional migration segment 1 [mm]	11.2674259	=$C$7*C13			
15		$t_{R\ segment\ 1}$	**4.41**	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	55
16		log $k_{F\ segment\ 1}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)		$tG_{segment\ 1}$	**4.41**
17		$k_{F\ segment\ 1}$	21.47535024	=10^C16		slope [°C/min]	12.5
19		**segment 2 (isothermal/isocratic)**				t_0	0.770
20		$b_{T\ segment\ 2}$	0	=$G$19*$G$23*$C$5/$G$24		T_{start}	115
21		$r_{segment\ 2}$	0.091297712			T_{final}	115
22		fractional migration segment 2 [mm]	6.847328395	=$C$7*C21			
23		$t_{R\ segment\ 2}$	**1.58**	=$G$19*$C$21*($C$17+1)		$\Delta T_{segment\ 2}$	0
24		log $k_{F\ segment\ 2}$	1.331940255	=C16		$tG_{segment\ 2}$	**1.58**
25		$k_{F\ segment\ 2}$	21.47535024	=C17		slope [°C/min]	0.0
27		**segment 3**				t_0	0.770
28		$b_{T\ segment\ 3}$	0.084998229	=($G$27*$G$31*$C$5/$G$32)		T_{start}	115
29		$r_{segment\ 3}$	0.758469943			T_{final}	180
30		fractional migration segment 3 [mm]	56.8852457	=$C$7*C29			
31		$t_{R\ segment\ 3}$	**6.001102041**	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25))		$\Delta T_{segment\ 3}$	65
32		log $k_{F\ segment\ 3}$	0.669494744	=LOG10($C$6)-($C$12*$C$15/$G$11)-($C$28*$C$31/$G$27)		$tG_{segment\ 3}$	**5.62**
33		$k_{F\ segment\ 3}$	4.671912968	=10^C32		slope [°C/min]	11.6
35		**segment 4 (isothermal/isocratic)**				t_0	0.770
36		$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)		T_{start}	180
37		$r_{segment\ 4}$	0	=(1-C13-C21-C29)		T_{final}	180
38		fractional migration segment 1 [mm]	0	=$C$7*C37			
39		$t_{R\ segment\ 4}$	**0**	=$G$35*$C$37*($C$33+1)		$\Delta T_{segment\ 4}$	0
40		log $k_{F\ segment\ 4}$	0.669494744	=C32		$tG_{segment\ 4}$	**1**
41		$k_{F\ segment\ 4}$	4.671912968	=10^C40		slope [°C/min]	0.0

Data_fitting_sulfamethazine | Data_fitting_total | **Figure_4a_sulfamethazine** | Figure_4a_total | calculation k0 at 100 °C | Figure_4b_sulfamethazine | Figure_4b_total | calci

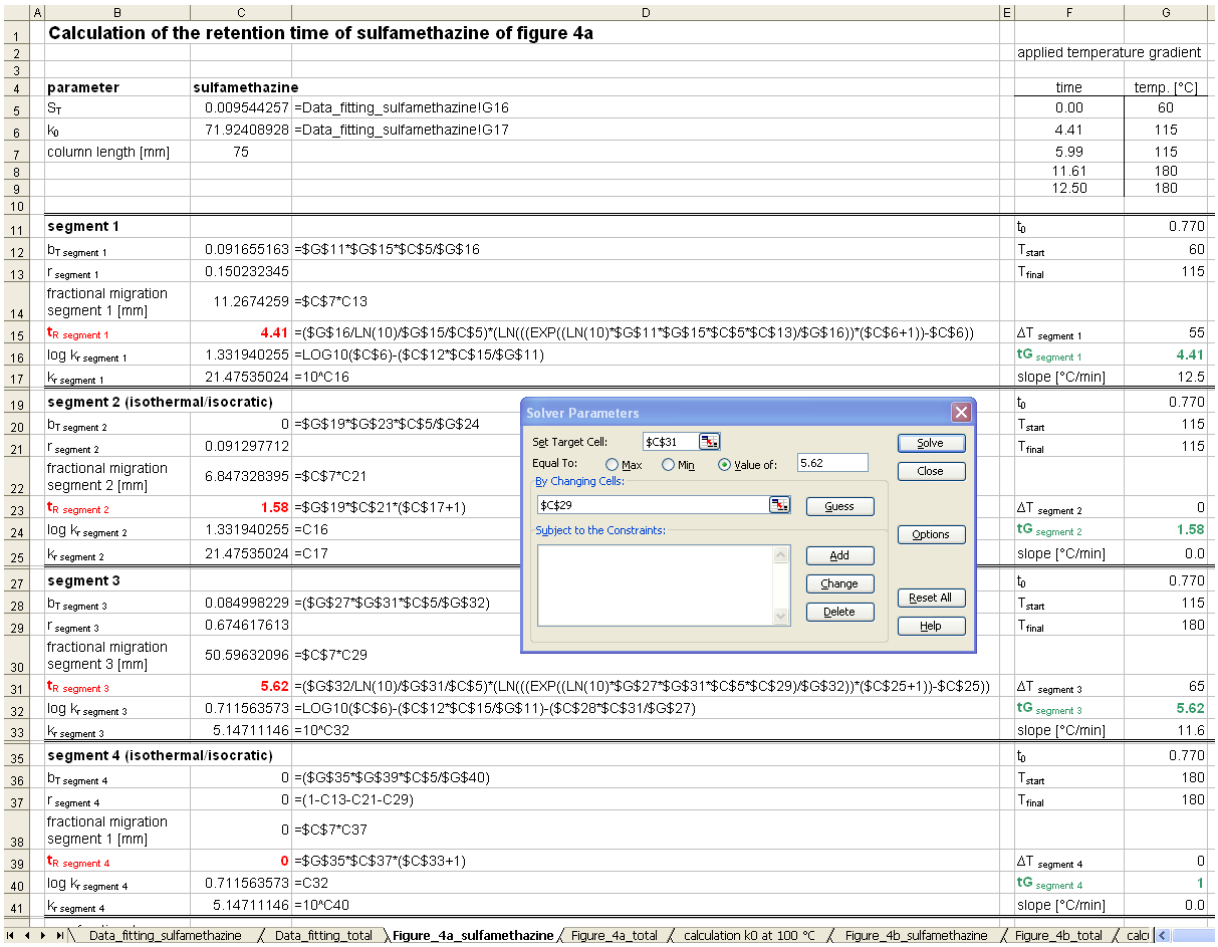
Figure A 3-6: Spreadsheet calculator, sheet displaying the equations that are needed to calculate the retention time and fractional migration of sulfamethazine for the third temperature segment.

The calculation starts again with the assumption that sulfamethazine will elute during the third segment of the gradient by setting the value of the fractional migration $r_{segment\,3}$ to 0.7585

($1 - r_{segment\,1} - r_{segment\,2} = 0.7585$), yielding a retention time of 6.00 min (cell: C31) for sulfamethazine for the third temperature segment.

Also in this case, the retention time of sulfamethazine is higher than the temperature-gradient time ($tG_{segment\,3}$) of the segment which was set to 5.62 min. Therefore, sulfamethazine does not elute during the third temperature segment and the value of the fractional migration has to be changed yielding a retention time of 5.62 min for sulfamethazine. Figure A 3-7 represents the required equations and Solver Parameters. The "Target Cell" corresponds with the retention time of sulfamethazine for the third segment (cell: C31) and should be set "Equal To" a "Value of" 5.62 "By Changing Cell" that corresponds with the fractional migration (cell: C29) of sulfamethazine during the third segment. Following, the solver function will

search for a value of the fractional migration ($r_{segment3}$) yielding a retention time for sulfamethazine of 5.62 min. A value of approximately 0.6746 gives the desired retention time corresponding to a migration distance of sulfamethazine of 50.6 mm (cell: C30) of the total column length for the third temperature segment.



	A	B	C	D	E	F	G
1		**Calculation of the retention time of sulfamethazine of figure 4a**					
2						applied temperature gradient	
3							
4		**parameter**	**sulfamethazine**			time	temp. [°C]
5		S_T	0.009544257	=Data_fitting_sulfamethazine!G16		0.00	60
6		k_0	71.92408928	=Data_fitting_sulfamethazine!G17		4.41	115
7		column length [mm]	75			5.99	115
8						11.61	180
9						12.50	180
10							
11		**segment 1**				t_0	0.770
12		$b_{T\ segment\ 1}$	0.091655163	=$G$11*$G$15*$C$5/$G$16		T_{start}	60
13		$r_{segment\ 1}$	0.150232345			T_{final}	115
14		fractional migration segment 1 [mm]	11.2674259	=$C$7*C13			
15		$t_{R\ segment\ 1}$	4.41	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	55
16		log $k_{f\ segment\ 1}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)		$tG_{segment\ 1}$	4.41
17		$k_{f\ segment\ 1}$	21.47535024	=10^C16		slope [°C/min]	12.5
19		**segment 2 (isothermal/isocratic)**				t_0	0.770
20		$b_{T\ segment\ 2}$	0	=$G$19*$G$23*$C$5/$G$24		T_{start}	115
21		$r_{segment\ 2}$	0.091297712			T_{final}	115
22		fractional migration segment 2 [mm]	6.847328395	=$C$7*C21			
23		$t_{R\ segment\ 2}$	1.58	=$G$19*$C$21*($C$17+1)		$\Delta T_{segment\ 2}$	0
24		log $k_{f\ segment\ 2}$	1.331940255	=C16		$tG_{segment\ 2}$	1.58
25		$k_{f\ segment\ 2}$	21.47535024	=C17		slope [°C/min]	0.0
27		**segment 3**				t_0	0.770
28		$b_{T\ segment\ 3}$	0.084998229	=($G$27*$G$31*$C$5/$G$32)		T_{start}	115
29		$r_{segment\ 3}$	0.674617613			T_{final}	180
30		fractional migration segment 3 [mm]	50.59632096	=$C$7*C29			
31		$t_{R\ segment\ 3}$	5.62	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C29)/$G$32))*($C$25+1))-$C$25))		$\Delta T_{segment\ 3}$	65
32		log $k_{f\ segment\ 3}$	0.711563573	=LOG10($C$6)-($C$12*$C$15/$G$11)-($C$28*$C$31/$G$27)		$tG_{segment\ 3}$	5.62
33		$k_{f\ segment\ 3}$	5.14711146	=10^C32		slope [°C/min]	11.6
35		**segment 4 (isothermal/isocratic)**				t_0	0.770
36		$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)		T_{start}	180
37		$r_{segment\ 4}$	0	=(1-C13-C21-C29)		T_{final}	180
38		fractional migration segment 1 [mm]	0	=$C$7*C37			
39		$t_{R\ segment\ 4}$	0	=$G$35*$C$37*($C$33+1)		$\Delta T_{segment\ 4}$	0
40		log $k_{f\ segment\ 4}$	0.711563573	=C32		$tG_{segment\ 4}$	1
41		$k_{f\ segment\ 4}$	5.14711146	=10^C40		slope [°C/min]	0.0

Solver Parameters: Set Target Cell: $C$31; Equal To: Max / Min / Value of: 5.62; By Changing Cells: $C$29; Subject to the Constraints:; Solve, Close, Guess, Options, Add, Change, Delete, Reset All, Help

Data_fitting_sulfamethazine | Data_fitting_total | Figure_4a_sulfamethazine | Figure_4a_total | calculation k0 at 100 °C | Figure_4b_sulfamethazine | Figure_4b_total | calci

Figure A 3-7: Spreadsheet calculator, sheet displaying the equations and Solver Parameters that are needed to calculate the fractional migration of sulfamethazine for the third temperature segment.

Finally, the value of the retention factor (k_0) of sulfamethazine corresponding to the final temperature of the third segment which is equal to the start temperature of the fourth temperature segment has to be calculated according to equation A 3-5 and yields a value of 5.147 (cell: C33).

Appendix 3-2.5. Calculation of the Retention Time of Sulfamethazine for the Fourth Temperature Segment of the Separation shown in Figure 3-4 a

The fourth segment of the temperature-gradient method shown in Figure 3-4 a is an isothermal/isocratic hold. Therefore, the retention time of sulfamethazine has to be calculated according to equation A 3-6 instead of equation A 3-4.

First, a number of experimental/theoretical data has to be determined and specified in the spreadsheet calculator (Figure A 3-8).

- the column dead time t_0 (cell: G35)
- the start temperature of the temperature gradient T_{start} (cell: G36)
- the final temperature of the temperature gradient T_{final} (cell: G37)
- the change in temperature during the temperature segment (cell: G39)
- the gradient time of the temperature segment tG_4 (cell: G40)
- a cell where the temperature-gradient steepness parameter b_T will be calculated according to equation A 3-2 (cell: C36)
- a cell for the fractional migration $r_{segment\,4}$ of the analyte during the second temperature segment (cell: C37)
- a cell containing the value of S_T (cell: C5)
- a cell containing the value of k_0 corresponding to the value of the retention factor calculated after the third temperature segment (cell: C33)
- a cell where the retention time will be calculated according to equation A 3-6 (cell: C39)
- a cell where the change of the retention factor during the fourth segment will be calculated according to equation A 3-5 (cells C40 and C41)

Figure A 3-8 represents a screenshot containing the equations which are needed to calculate the retention time and fractional migration of sulfamethazine during the fourth segment of the temperature-gradient method.

	A	B	C	D	E	F	G
4		**parameter**	**sulfamethazine**			time	temp. [°C]
5		S_T	0.009544257	=Data_fitting_sulfamethazine!G16		0.00	60
6		k_0	71.92408928	=Data_fitting_sulfamethazine!G17		4.41	115
7		column length [mm]	75			5.99	115
8						11.61	180
9						12.50	180
10							
11		**segment 1**				t_0	0.770
12		$b_{T\ segment\ 1}$	0.091655163	=$G$11*$G$15*$C$5/$G$16		T_{start}	60
13		$r_{segment\ 1}$	0.150232345			T_{final}	115
14		fractional migration segment 1 [mm]	11.2674259	=$C$7*C13			
15		$t_{R\ segment\ 1}$	4.41	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	55
16		log $k_{F\ segment\ 1}$	1.331940255	=LOG10($C$6)-($C$12*$C$15/$G$11)		$tG_{segment\ 1}$	4.41
17		$k_{F\ segment\ 1}$	21.47535024	=10^C16		slope [°C/min]	12.5
19		**segment 2 (isothermal/isocratic)**				t_0	0.770
20		$b_{T\ segment\ 2}$	0	=$G$19*$G$23*$C$5/$G$24		T_{start}	115
21		$r_{segment\ 2}$	0.091297712			T_{final}	115
22		fractional migration segment 2 [mm]	6.847328395	=$C$7*C21			
23		$t_{R\ segment\ 2}$	1.58	=$G$19*$C$21*($C$17+1)		$\Delta T_{segment\ 2}$	0
24		log $k_{F\ segment\ 2}$	1.331940255	=C16		$tG_{segment\ 2}$	1.58
25		$k_{F\ segment\ 2}$	21.47535024	=C17		slope [°C/min]	0.0
27		**segment 3**				t_0	0.770
28		$b_{T\ segment\ 3}$	0.084998229	=($G$27*$G$31*$C$5/$G$32)		T_{start}	115
29		$r_{segment\ 3}$	0.674617613			T_{final}	180
30		fractional migration segment 3 [mm]	50.59632096	=$C$7*C29			
31		$t_{R\ segment\ 3}$	5.62	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25))		$\Delta T_{segment\ 3}$	65
32		log $k_{F\ segment\ 3}$	0.711563573	=LOG10($C$6)-($C$12*$C$15/$G$11)-($C$28*$C$31/$G$27)		$tG_{segment\ 3}$	5.62
33		$k_{F\ segment\ 3}$	5.14711146	=10^C32		slope [°C/min]	11.6
35		**segment 4 (isothermal/isocratic)**				t_0	0.770
36		$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)		T_{start}	180
37		$r_{segment\ 4}$	0.08385233	=(1-C13-C21-C29)		T_{final}	180
38		fractional migration segment 1 [mm]	6.288924747	=$C$7*C37			
39		$t_{R\ segment\ 4}$	0.396896206	=$G$35*$C$37*($C$33+1)		$\Delta T_{segment\ 4}$	0
40		log $k_{F\ segment\ 4}$	0.711563573	=C32		$tG_{segment\ 4}$	1
41		$k_{F\ segment\ 4}$	5.14711146	=10^C40		slope [°C/min]	0.0
43		sum fractional migration [mm]	75	=C14+C22+C30+C38			
44		$t_{R\ total}$	12.01	=C39+C31+C23+C15			

Data_fitting_sulfamethazine | Data_fitting_total | **Figure_4a_sulfamethazine** | Figure_4a_total | calculation k0 at 100 °C | Figure_4b_sulfamethazine | Figure_4b_total | calci

Figure A 3-8: Spreadsheet calculator, sheet displaying the equations that are needed to calculate the retention time and fractional migration of sulfamethazine for the fourth temperature segment.

The calculation starts by setting the value of the fractional migration $r_{segment\,4}$ of sulfamethazine to 0.08385 ($1-r_{segment1}-r_{segment2}-r_{segment3}=0.08385$). This value yields a retention time of 0.397 min (cell: C39) for sulfamethazine for the fourth segment. As can be seen, this value is smaller than the temperature-gradient time of 1 min (cell: G40) and points out that sulfamethazine elutes during the fourth segment from the column.

Finally, the retention times of sulfamethazine during each segment sum up to 12.01 min (cell: C44), which represents the total retention of sulfamethazine.

Appendix 3-3. Calculation of the Retention Time of Sulfamethazine for the Separation shown in Figure 3-4 b

To calculate the retention times of selected sulfonamides for the separation shown in Figure 3-4 b, values of the retention factors corresponding to the selected start temperature of the gradient of 100 °C were required. Therefore, a linear regression was performed using isothermal/isocratic data at 60 °C and 180 °C. The experimental data are represented in Table A 3-2.

Table A 3-2: Overview of experimentally obtained isothermal/isocratic retention times of selected sulfonamides at 60 °C and 180 °C.

analyte	experimental retention time [min] at 60 °C (333.16 K)	experimental retention time [min] at 180 °C (453.15 K)
uracil	1.090	0.771
sulfadiazine	8.906	1.299
sulfathiazole	12.740	1.195
sulfamerazine	21.921	1.922
sulfamethoxazole	51.120	2.087
sulfamethazine	54.608	3.157

On the basis of these data, values of the retention factor (k_0) corresponding to 100 °C were calculated for each sulfonamide and have been employed as start values for the prediction of the retention times of these compounds for the method shown in Figure 3-4 b.

Afterwards, the retention time of each sulfonamide was calculated using the same approach as described in Appendix 3-2.

List of Symbols of Appendix for Chapter 3

b_T	temperature-gradient steepness parameter
k_0	retention factor of an analyte i at the start of the temperature gradient
r	fractional migration of an analyte i during a segment of the temperature-gradient method
S_T	analyte specific constant of the LES retention model
ΔT	difference between start and final temperature of the temperature gradient
t_0	column dead time
T_{final}	final temperature of the temperature gradient
tG	temperature-gradient time
t_R	retention time of an analyte i
T_{start}	start temperature of the temperature gradient

Chapter 4. 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*

*Submitted to Journal of Chromatography A, in revision

4.1 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 15 °C up to 75 °C (ΔT = 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 retention time 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 °C to 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.

4.2 Experimental Section

4.2.1 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.

4.2.2 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 × 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.

4.2.3 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.

4.2.4 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.

4.2.5 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 8 °C min^{-1} were applied. Table 4-1 summarizes the temperature-gradient measurements which have been employed as input data.

Table 4-1: 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		

4.3 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 equations 4-1 and 4-2 [33,34].

$$t_R = \frac{t_0}{2.3 b_T} \ln\left[e^{2.3 b_T} \left(k_0 + 1\right) - k_0 \right] \tag{4-1}$$

$$\text{with } b_T = \frac{t_0 S_T \Delta T}{tG} \tag{4-2}$$

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 equations 4-1 and 4-2. 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 equation 4-3 [33,39,40].

$$t_R = \frac{t_0}{2.3 b_T} \ln\left[e^{2.3 b_T r} \left(k_0 + 1\right) - k_0 \right] \tag{4-3}$$

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

$$t_R = rt_0\left(k_0 + 1\right) \tag{4-4}$$

The sum of the fractional migration r of an analyte across the column during each temperature segment can be written as ($r_1 + r_2 + ...r_n = 1$). Moreover, equation 4-5 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 employed instead of k_0 in equation 4-3.

$$\log k_r = \log k_0 - \frac{b_T t_R}{t_0} \tag{4-5}$$

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 Appendix for Chapter 4.

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 retention times of 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 ($\Delta T, tG$). 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].

4.4 Results and Discussion

4.4.1 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 4-1). 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 (equations 4-1 and 4-2). Afterwards, the calculated values of k_0 were employed to represent a plot of $\ln k_0$ vs T which is shown in Figure 4-1 a. The corresponding plot of $\ln k$ vs T based on isothermal measurements is shown in Figure 4-1 b.

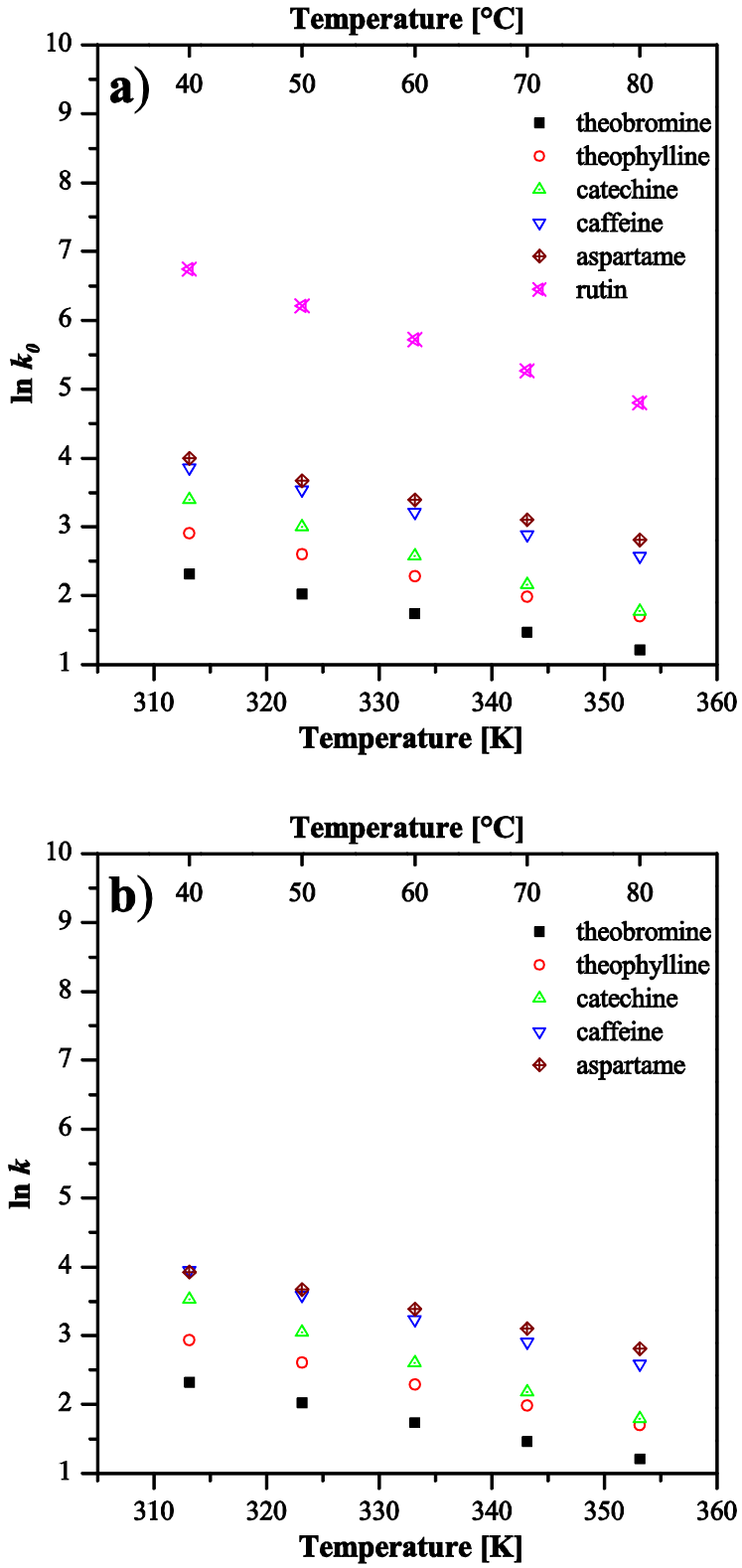


Figure 4-1: Comparison of different plots of $\ln k$ vs T. (a) calculated values of $\ln k_0$ based on temperature gradients, (b) calculated values of $\ln k$ based on isothermal measurements. Chromatographic conditions: stationary phase: Waters XBridge C_{18} (50 × 3.0 mm, 3.5 µm); mobile phase: water + 0.1% formic acid; injection volume: 4 µL, see also experimental sections 4.2.4 and 4.2.5.

As can be seen, both plots look very similar and show a strict linear relationship between the natural logarithm of the retention factor versus temperature for each food additive. This is confirmed by the data given in Table 4-2 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.

Table 4-2: Overview of characteristics of linear regression of the plots shown in Figure 4-1.

figure	parameter	theobromine	theophylline	catechine	caffeine	aspartame	rutin
4-1 a	slope [$\times 10^{-2}$]	–2.75	–3.02	–4.08	–3.21	–2.94	–4.80
	intercept [$\times 10^{1}$]	1.09	1.24	1.62	1.39	1.32	2.18
	R^2	0.9994	0.9997	0.9998	0.9999	0.9993	0.9989
4-1 b	slope [$\times 10^{-2}$]	–2.82	–3.03	–4.24	–3.20	–2.61	---
	intercept [$\times 10^{1}$]	1.06	1.19	1.62	1.34	1.16	---
	R^2	0.9973	0.9981	0.9989	0.9991	0.9998	---

Moreover, Figure 4-1 a 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 six hours 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 4-3 reveals a comparison of relative errors of interpolated (50 °C - 70 °C) and extrapolated (90 °C - 120 °C) isothermal retention times of selected food additives.

Table 4-3: 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*

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 4-1) 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 4-1) 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 4-3).

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 4-3 underline that isothermal predictions based on isothermal input data should only be applied for a small temperature interval of e.g., ΔT = 40 °C when

using only two temperatures for data fitting. To predict isothermal retention times using a larger temperature interval of e.g., ΔT = 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 Appendix for Chapter 4.

4.4.2 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 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 4-1). 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 (equations 4-1 and 4-2). Afterwards, the calculated values of S_T were employed to plot S_T vs T which is shown in Figure 4-2.

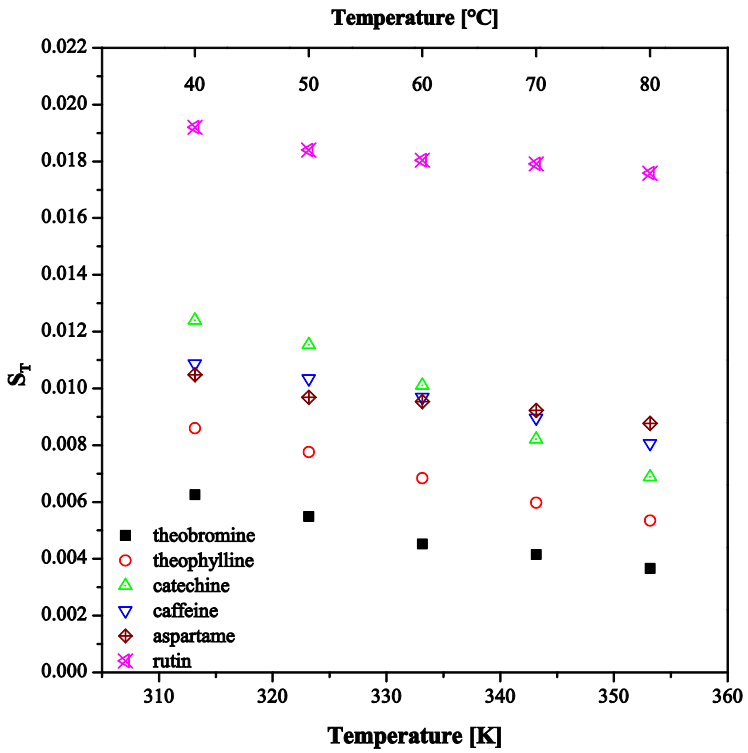


Figure 4-2: Plot of S_T vs T of six food additives. Calculated values of S_T based on experimental temperature-gradient measurements. For chromatographic conditions see section 4.2.5.

Figure 4-2 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 4-4 where characteristics of the linear regression of the S_T vs T plot are represented for each food additive.

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

parameter	theobromine	theophylline	catechine	caffeine	aspartame	rutin
slope [$\times 10^{-5}$]	-6.55	-8.27	-14.37	-7.00	-3.89	-3.72
intercept [$\times 10^{-2}$]	2.66	3.40	5.78	3.29	2.25	3.06
R^2	0.9720	0.9960	0.9864	0.9899	0.9462	0.9070

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 4-4 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 gradient runs 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 4-1) were employed to calculate S_T as well as k_0 depending on temperature by means of linear regression of the S_T vs T and the $\ln k_0$ 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 4-5. 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 4-5.

Table 4-5: 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*

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 Figure 4-1 a and Table 4-5, 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].

4.4.3 Systematic Temperature-Programming Method Development

The new experimental design has been employed to perform systematic temperature-programming method development of 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 °C to 140 °C with a slope of 2 °C min^{-1} (run 1 of Table 4-1)
- 40 °C to 140 °C with a slope of 6 °C min^{-1} (run 3 of Table 4-1)
- 80 °C to 180 °C with a slope of 2 °C min^{-1} (run 17 of Table 4-1)
- 80 °C to 180 °C with a slope of 6 °C min^{-1} (run 19 of Table 4-1)

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 Figure 4-3, where the start temperature for the gradients ranged from 40 °C to 70 °C.

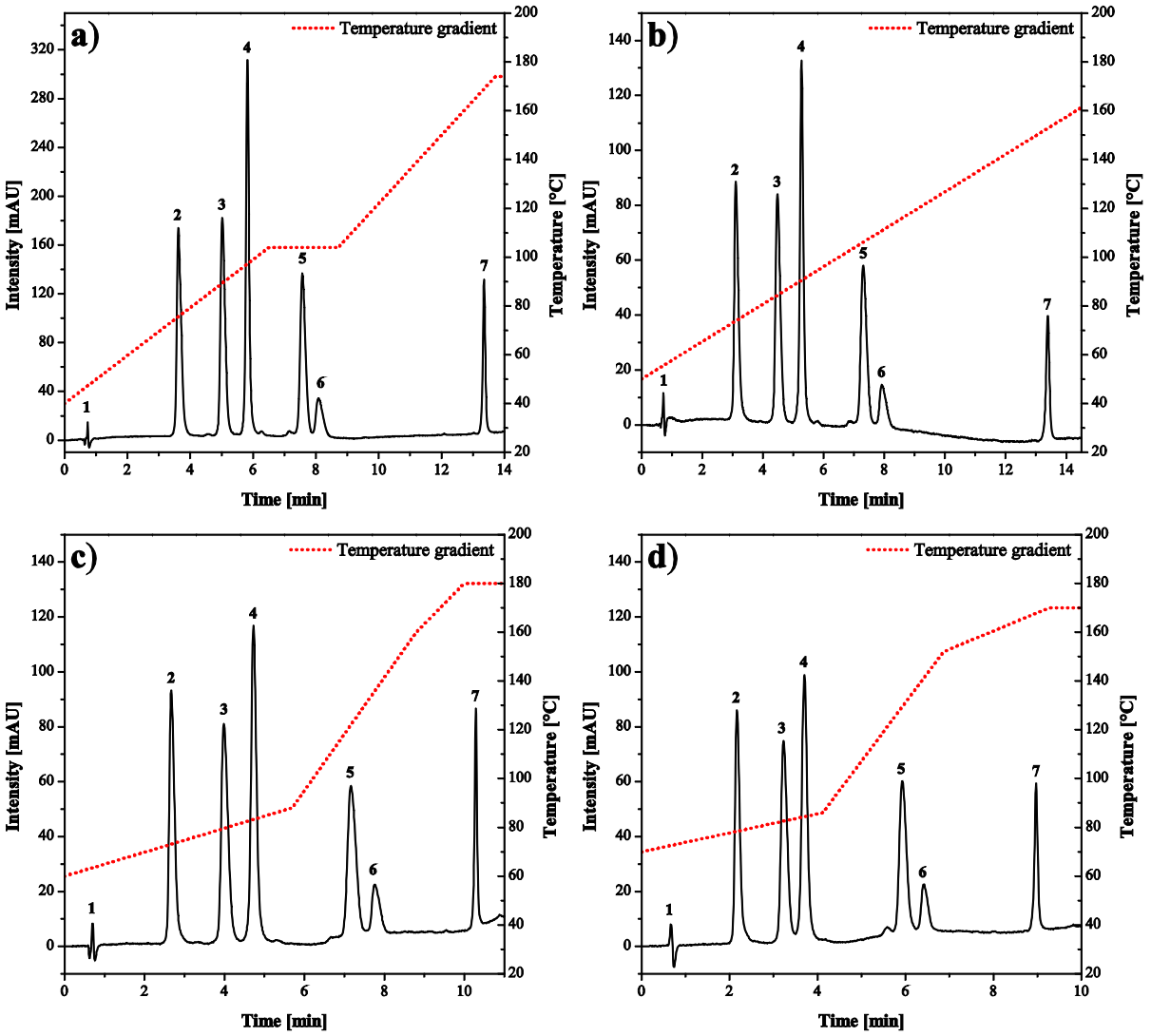


Figure 4-3: Chromatograms of the separation of six food additives by temperature-gradient elution. Different start temperatures of the gradient were employed: (a) = 40 °C, (b) = 50 °C, (c) = 60 °C, (d) = 70 °C. Chromatographic conditions: Waters XBridge C_{18} (50 × 3.0 mm, 3.5 µm); mobile phase: water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

Moreover, in Table 4-6 predicted and experimental retention times, relative errors and average relative errors are compared. In the case of a simple linear temperature gradient (Figure 4-3 b) 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 Figure 4-3 a, 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 Figure 4-3 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 (Figure 4-3 a and d) or fourth (Figure 4-3 c) 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 (Figure 4-3 b) a significantly lower relative error of predicted retention time of 1.1% is observed for rutin.

Table 4-6: 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 Figure 4-3.

figure	analytes	seg. [a]	peak width [min] [b]	expt. RT [min]	pred. RT [min]	difference [min]	relative error [%]	average rel. error [%]
4-3 a	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	
4-3 b	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	
4-3 c	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	
4-3 d	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.

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 (Figure 4-3 b) the food additives can be separated within approximately 14 minutes 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 (Figure 4-3 d), the analytes were separated within 9 minutes with a critical resolution between peaks 5/6 of 1.61. The critical resolution between peaks 5/6 of the methods shown in Figure 4-3 a 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 Figure 4-3 d should increase from 1.61 to 2.27, but it has to be considered that the analysis time would also be doubled.

4.4.4 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 4.4.3 (Figure 4-3 d). 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.

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

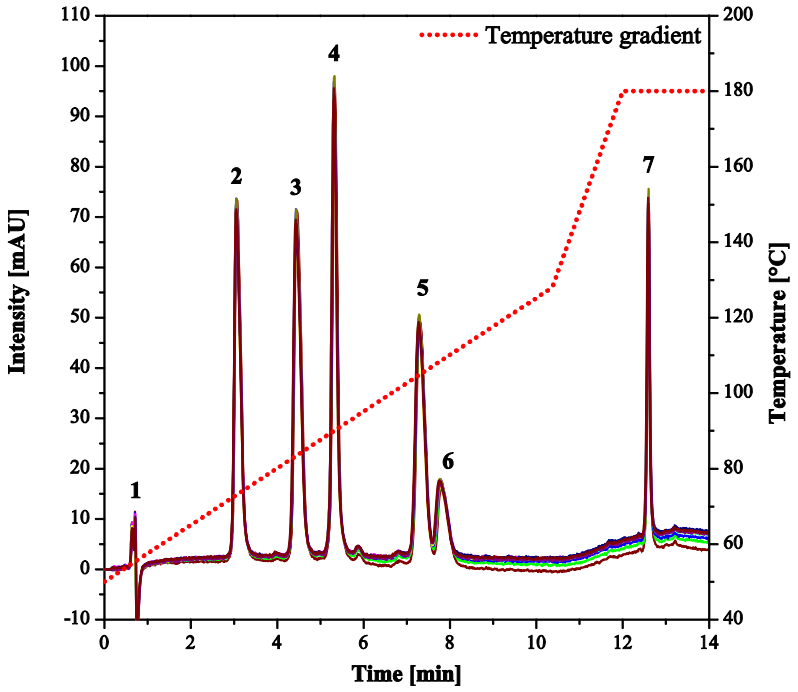


Figure 4-4: Overlay of nine chromatograms of the separation of six food additives and uracil by temperature-gradient elution. Chromatographic conditions: Waters XBridge C_{18} (50 × 3.0 mm, 3.5 µm); mobile phase: water + 0.1% formic acid; flow rate: 0.5 mL min^{-1}; injection volume: 1 µL; temperature 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, 7) rutin.

As can be seen, there are only marginal differences between the nine chromatograms which is also underlined by the statistical data given in Table 4-7.

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

analyte	retention time [min]	standard deviation [min]	relative standard deviation (RSD) [%]
theobromine	3.05	0.01	0.19
theophylline	4.44	0.01	0.18
catechine	5.31	0.01	0.15
caffeine	7.28	0.01	0.14
aspartame	7.77	0.02	0.23
rutin	12.59	0.01	0.05

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 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 4.4.3). 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 (R_S) using the same method which has been employed for the evaluation of the repeatability. Table 4-8 shows a comparison of the critical resolution between caffeine and aspartame when the temperatures of the gradient points were changed by ± 2 °C.

Table 4-8: Change of the critical resolution (R_S) between caffeine and aspartame when varying the temperature of the gradient points based on the method depicted in Figure 4-4.

	$\Delta T = -2$ °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
R_S	1.06		1.10		1.13		1.09		1.15	

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 4-8 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.

Regarding the temperature-gradient method preferred for the separation of the food additives discussed in section 4.4.3 (Figure 4-3 d), 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.

4.4.5 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 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 minutes (2 °C min^{-1}) is applied, the last eluting compound should be eluted from the column within 50 minutes. 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 Figure 4-5.

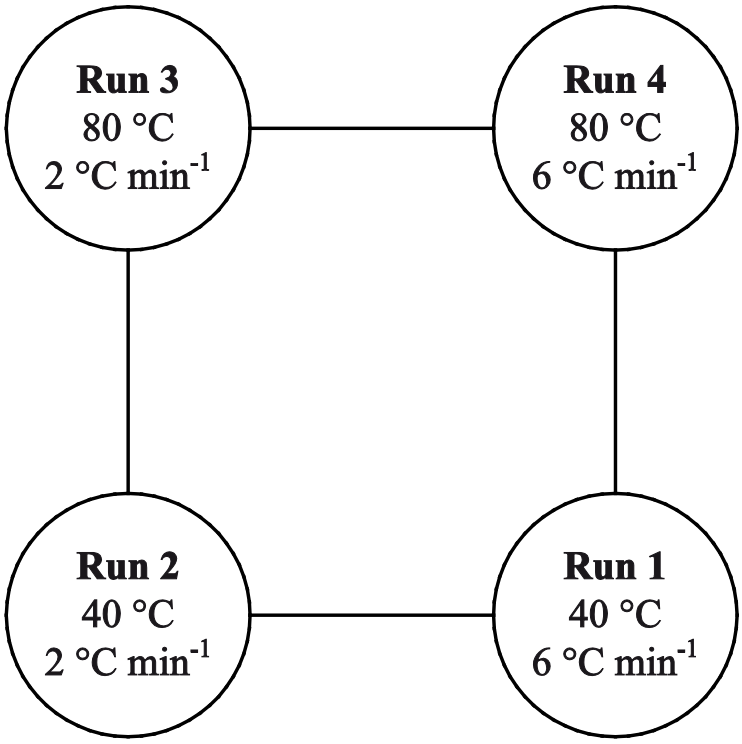


Figure 4-5: Recommended experimental design to perform systematic temperature-programming method development by means of the LES model in high-temperature liquid chromatography.

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 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.

4.5 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.

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Appendix for Chapter 4

Abstract

The Appendix for Chapter 4 contains additional information on how the parameters S_T and k_0 of the LES retention model were calculated depending on temperature. Additional Figures as well as Tables are given that will help understanding Chapter 4.

Table of Contents

Eight Figures and ten Tables.

Appendix 4-1. Determination of the Model Parameters S_T and k_0

For the determination of the parameters S_T and k_0 of the LES model equations A 4-1 and A 4-2 are required.

$$t_R = \frac{t_0}{2.3 b_T} \ln\left[e^{2.3 b_T} \left(k_0 + 1\right) - k_0 \right] \quad \text{(A 4-1)}$$

$$b_T = \frac{t_0 S_T \Delta T}{tG} \quad \text{(A 4-2)}$$

The combination of both equations yields

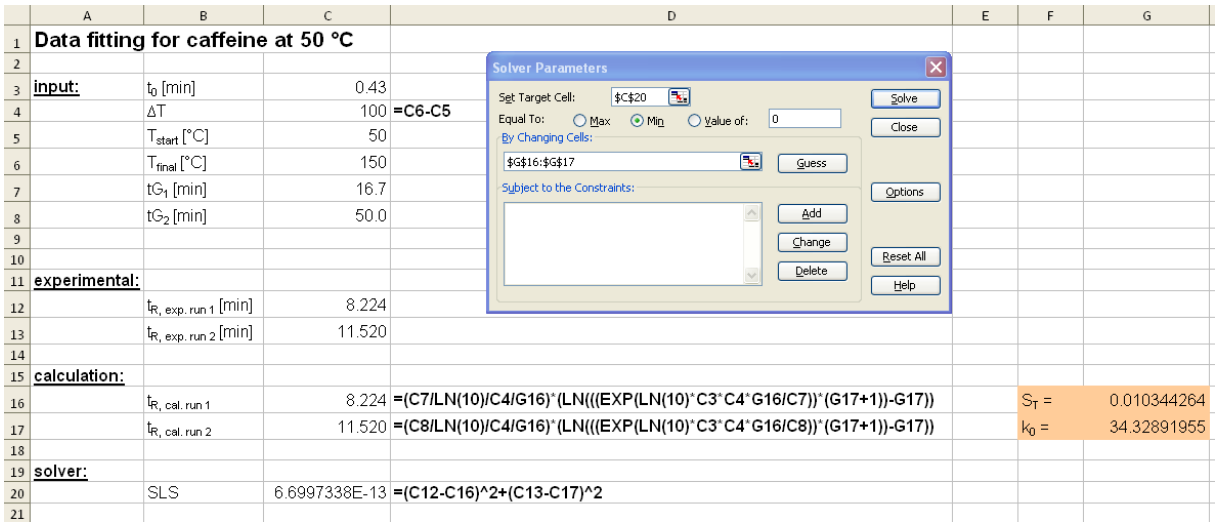
$$t_R = \frac{tG}{2.3 \Delta T S_T} \ln\left[e^{\frac{2.3 t_0 \Delta T S_T}{tG}} \left(k_0 + 1\right) - k_0 \right] \quad \text{(A 4-3)}$$

Using equation A 4-3, at least two temperature-gradient input measurements are required. These runs should differ in the gradient slope by a factor of three. Moreover, selected analytes should elute during the applied temperature gradient. Table A 4-1 contains experimentally obtained retention times of selected food additives for two temperature-gradient measurements from 50 °C to 150 °C within 16.67 minutes (6 °C min^{-1}) and 50 minutes (2 °C min^{-1}).

Table A 4-1: Overview of experimentally obtained retention times of selected food additives of the basic input measurements. Temperature interval 50 °C - 150 °C.

analyte	experimental retention time [min], 2 °C min^{-1}	experimental retention time [min], 6 °C min^{-1}
theobromine	3.296	3.541
theophylline	4.843	5.643
catechine	5.728	7.456
caffeine	8.224	11.520
aspartame	9.088	12.885
rutin	16.011	35.051

The numerical solution of equation A 4-3 can be achieved using a spreadsheet calculator such as Microsoft Excel Solver. Figure A 4-1 represents a screenshot of the spreadsheet calculator.



	A	B	C	D	E	F	G
1	**Data fitting for caffeine at 50 °C**						
2							
3	**input:**	t_0 [min]	0.43				
4		ΔT	100	=C6-C5			
5		T_{start} [°C]	50				
6		T_{final} [°C]	150				
7		tG_1 [min]	16.7				
8		tG_2 [min]	50.0				
9							
10							
11	**experimental:**						
12		$t_{R,\ exp.\ run\ 1}$ [min]	8.224				
13		$t_{R,\ exp.\ run\ 2}$ [min]	11.520				
14							
15	**calculation:**						
16		$t_{R,\ cal.\ run\ 1}$	8.224	=(C7/LN(10)/C4/G16)*(LN(((EXP(LN(10)*C3*C4*G16/C7))*(G17+1))-G17))		S_T =	0.010344264
17		$t_{R,\ cal.\ run\ 2}$	11.520	=(C8/LN(10)/C4/G16)*(LN(((EXP(LN(10)*C3*C4*G16/C8))*(G17+1))-G17))		k_0 =	34.32891955
18							
19	**solver:**						
20		SLS	6.6997338E-13	=(C12-C16)^2+(C13-C17)^2			
21							

Figure A 4-1: Spreadsheet calculator, sheet displaying the equations Solver Parameters that are needed to calculate S_T and k_0.

Input:

A number of experimental data have to be determined and specified.

- the column dead time (t_0)
- the start temperature of the temperature gradient (T_{start})
- the final temperature of the temperature gradient (T_{final})
- the change in temperature during the temperature gradient ($\Delta T = T_{final} - T_{start}$)
- the gradient time of the first temperature gradient (tG_1)
- the gradient time of the second temperature gradient (tG_2)

Moreover, two additional cells have to be defined for S_T and k_0.

Experimental:

The experimentally obtained retention times of the selected analyte (rutin) for the first and the second temperature gradient have to be specified in the spreadsheet calculator.

- experimentally obtained retention time for the first gradient ($t_{R,\exp.run1}$[min])
- experimentally obtained retention time for the second gradient ($t_{R,\exp.run2}$[min])

Calculation:

Here, two cells have to be specified where the theoretical retention times of the selected analyte (rutin) are calculated according to equation A 4-3 for the first and second temperature gradient.

- theoretical retention for the first gradient ($t_{R,cal.run1}$ [min])
- theoretical retention for the first gradient ($t_{R,cal.run2}$ [min])

Solver:

Furthermore, a cell is implemented where the sum of the least squares (SLS) is calculated, representing the differences between calculated and experimental retention times for the first and second temperature gradient.

$$SLS = \left(t_{R,cal.run1} - t_{R,\exp.run1}\right)^2 + \left(t_{R,cal.run2} - t_{R,\exp.run2}\right)^2$$

Microsoft Excel Solver is then employed to calculate the values of S_T and k_0 for the selected analyte depending on the sum of the least squares being as small as possible. The "Target Cell" is the cell which corresponds to the SLS and should be set "Equal To: Min" or a "Value of" 0. The cells which are corresponding to S_T and k_0 are the cells used by MS Excel Solver to solve the system of equations. Following, the Solver function will search for values of S_T and k_0 according to equation A 4-3 yielding the same retention times as those obtained experimentally. The described procedure has to be performed for each analyte in the sample mixture.

Appendix 4-2. Temperature-Dependent Calculation of the Retention Factor k_0

To calculate the parameter k_0 depending on temperature, a data set of ten temperature-gradient input runs was employed. For these measurements the start temperature of the gradients were set from 40 °C (313.15 K) up to 80 °C (353.15 K) with increments of 10 °C. The temperature-gradient slopes were set to 2 °C min^{-1} and 6 °C min^{-1} for each start temperature (runs 1, 2, 5, 7, 9, 11, 13, 15, 17, and 19 of Table 4-1). Afterwards, data fitting as described in Appendix 4-1 was performed for each food additive. These calculations yield values of k_0 for different start temperatures for each food additive and were depicted in Table A 4-2.

Table A 4-2: Overview of calculated retention factors (k_0) of selected food additives for different start temperatures of the basic input measurements.

start temperature [°C]	start temperature [K]	k_0 theobromine	k_0 theophylline	k_0 catechine	k_0 caffeine	k_0 aspartame	k_0 rutin
40	313.15	10.08	18.28	29.79	47.29	54.28	848
50	323.15	7.57	13.43	20.04	34.33	39.12	497
60	333.15	5.69	9.83	13.10	24.71	29.64	304
70	343.15	4.35	7.27	8.68	17.93	22.21	194
80	353.15	3.37	5.48	5.88	13.12	16.54	121

Afterwards, the obtained values of k_0 were employed to calculate values of the natural logarithm of the retention factor ($\ln k_0$) for each food additive. Data are given in Table A 4-3.

Table A 4-3: Overview of calculated $\ln k_0$ values of selected food additives for different start temperatures of the basic input measurements.

start temperature [°C]	start temperature [K]	$\ln k_0$ theobromine	$\ln k_0$ theophylline	$\ln k_0$ catechine	$\ln k_0$ caffeine	$\ln k_0$ aspartame	$\ln k_0$ rutin
40	313.15	2.31	2.91	3.39	3.86	3.99	6.74
50	323.15	2.02	2.60	3.00	3.54	3.67	6.21
60	333.15	1.74	2.29	2.57	3.21	3.39	5.72
70	343.15	1.47	1.98	2.16	2.89	3.10	5.27
80	353.15	1.21	1.70	1.77	2.57	2.81	4.80

Finally, these values were employed to build a plot of $\ln k_0$ vs T which is shown in Figure 4-1 a.

Moreover, Table A 4-4 contains characteristics of linear regression of the $\ln k_0$ vs T plot of the food additives (T equals temperature in K).

Table A 4-4: Overview of characteristics of linear regression of the $\ln k_0$ vs T plot of the food additives. Data shown correspond to Figure 4-1 a of Chapter 4.

	theobromine	**theophylline**	**catechine**	**caffeine**	**aspartame**	**rutin**
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	0.9989	0.9989

In order to calculate the retention factors of the food additives depending on the start temperature using as few as possible basic input measurements, the retention factors or rather the natural logarithm of the retention factors ($\ln k_0$) obtained at 313.15 K (40 °C) and 353.15 K (80 °C) were employed. On the basis of these values linear equations were derived for each food additive and are presented in Table A 4-5.

Table A 4-5: Overview of slope and intercept of the linear equations of selected food additives, based on a plot of $\ln k_0$ vs T using data at 313.15 K and 353.15 K.

	theobromine	**theophylline**	**catechine**	**caffeine**	**aspartame**	**rutin**
slope	-2.74×10^{-2}	-3.01×10^{-2}	-4.05×10^{-2}	-3.21×10^{-2}	-2.97×10^{-2}	-4.86×10^{-2}
intercept	1.09×10^{1}	1.23×10^{1}	1.61×10^{1}	1.39×10^{1}	1.33×10^{1}	2.20×10^{1}

Following, the linear equations were used to calculate values of the retention factors of the analytes for different start temperatures of the temperature gradients. The interpolated retention factors are shown in Table A 4-6 for each food additive. Furthermore, these retention factors were employed as start values for the calculation of the retention times of the food additives which were shown in Figure 4-3, b, c, and d.

Table A 4-6: Calculated retention factors of food additives for different start temperatures of the temperature gradients.

start temperature [°C]	start temperature [K]	k_0 theobromine	k_0 theophylline	k_0 catechine	k_0 caffeine	k_0 aspartame	k_0 rutin
50	323.15	7.67	13.52	19.87	34.32	40.33	522
60	333.15	5.83	10.01	13.24	24.91	29.97	321
70	343.15	4.43	7.41	8.83	18.08	22.27	197

Appendix 4-3. Temperature-Dependent Calculation of S_T

To calculate the parameter S_T depending on temperature, a very similar approach was employed as for the temperature-dependent calculation of the parameter k_0 which was described in Appendix 4-2.

A data set of ten temperature-gradient input measurements was employed where the start temperature of the gradients were set from 40 °C (313.15 K) to 80 °C (393.15 K) with increments of 10 °C. The temperature-gradient slopes were set to 2 °C min^{-1} and 6 °C min^{-1} for each start temperature (runs 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19 of Table 4-1). Following, data fitting was performed using the approach described in Appendix 4-1 for each food additive at each start temperature of the temperature gradients. The obtained values of S_T are given in Table A 4-7. Moreover, the values of S_T were employed to build a plot of S_T vs T which is shown in Figure 4-2.

Table A 4-7: Overview of calculated values of S_T of the food additives for different start temperatures of the basic input measurements.

start temperature [°C]	start temperature [K]	S_T theobromine	S_T theophylline	S_T catechine	S_T caffeine	S_T aspartame	S_T rutin
40	313.15	0.00626	0.00860	0.01241	0.01087	0.01048	0.01921
50	323.15	0.00550	0.00777	0.01155	0.01034	0.00970	0.01840
60	333.15	0.00453	0.00684	0.01012	0.00970	0.00954	0.01804
70	343.15	0.00415	0.00598	0.00822	0.00896	0.00923	0.01792
80	353.15	0.00366	0.00536	0.00689	0.00806	0.00877	0.01759

Furthermore, Table A 4-8 contains data of linear regression of the S_T vs T plot of each food additive.

Table A 4-8: Overview of data of linear regression of the S_T vs T plot for each food additive. Data shown correspond to Figure 4-2.

	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

In order to calculate the parameter S_T of the food additives depending on the start temperature using as few as possible basic input measurements, the values of S_T obtained at 313.15 K (40 °C) and 353.15 K (80 °C) were employed. On the basis of these values a linear equation was built for each food additive which is represented in Table A 4-9.

Table A 4-9: Overview of the linear equations for selected food additives, based on a plot of S_T vs T using data at 313.15 K and 353.15 K.

	theobromine	**theophylline**	**catechine**	**caffeine**	**aspartame**	**rutin**
slope	-6.50×10^{-5}	-8.10×10^{-5}	-1.38×10^{-4}	-7.02×10^{-5}	-4.28×10^{-5}	-4.04×10^{-5}
intercept	2.66×10^{-2}	3.40×10^{-2}	5.57×10^{-2}	3.29×10^{-2}	2.39×10^{-2}	3.19×10^{-2}

Following, the linear equations were used to calculate values of S_T for the analytes at different start temperatures of the temperature gradients. The interpolated values of S_T are shown in Table A 4-10 for each food additive. Furthermore, these values of S_T were employed as start values for the calculation of the retention times of the food additives which were shown in Figure 4-3, b, c, and d of Chapter 4.

Table A 4-10: Calculated values of S_T for food additives for different start temperatures of the temperature gradient.

start temperature [°C]	**start temperature [K]**	S_T **theobromine**	S_T **theophylline**	S_T **catechine**	S_T **caffeine**	S_T **aspartame**	S_T **rutin**
50	323.15	0.00561	0.00779	0.01103	0.01017	0.01006	0.01880
60	333.15	0.00496	0.00698	0.00965	0.00947	0.00963	0.01840
70	343.15	0.00431	0.00617	0.00827	0.00877	0.00920	0.01799

Appendix 4-4. Isothermal Separation of the Food Additives

In HPLC the practitioner has the choice between isothermal or temperature-programming mode. Regarding an isothermal separation, Figure A 4-2 shows an isothermal resolution map of six food additives within a temperature range from 20 °C to 140 °C.

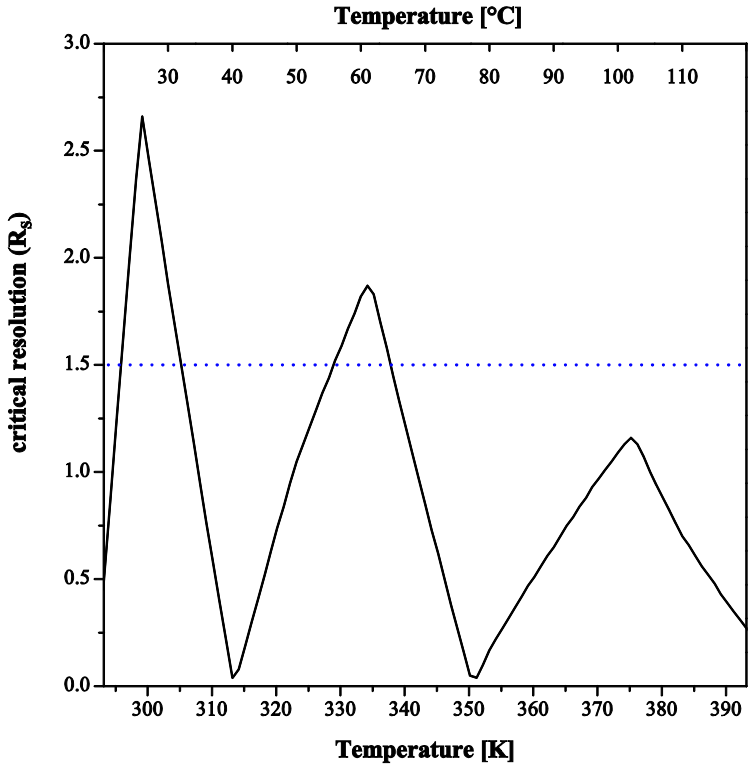


Figure A 4-2: Isothermal resolution map of selected food additives. Chromatographic conditions: stationary phase: Waters XBridge C_{18} (50 × 3.0 mm, 3.5 µm); mobile phase: deionized water + 0.1% formic acid; flow rate: 0.5 mL min^{-1}; temperature range: 40 °C to 120 °C; UV detection at 200 nm.

If a baseline separation ($R_S \geq 1.5$) of the analytes is absolutely mandatory, only two temperature intervals ranging from 23 °C to 32 °C and 56 °C to 65 °C would be suitable. From a practical point of view, a separation at a temperature of 27 °C should yield a high critical resolution of 2.7, but unreasonably long retention times will be expected. If the separation would be carried out at a temperature of 62 °C, a critical resolution of 1.9 might be achieved. In this case, the first five food additives will elute after approximately 18 minutes whereas rutin needs approximately two hours to elute from the column. Therefore, the food additives should be separated in temperature-programming mode which was discussed in section 4.4.3.

Appendix 4-5. Calculation of the Retention Time of Rutin for the Separation shown in Figure 4-3 c

Appendix 4-5.1 Required Equations

To calculate the retention time of an analyte for a segmented temperature gradient, equations A 4-4 to A 4-6 are needed. Equation A 4-4 describes the retention time of an analyte during a certain temperature segment. Equation A 4-5 can be employed to calculate the change of the retention factor of an analyte depending on the applied temperature gradient of a temperature segment. This value of the retention factor represents the start value of k_0 for the next temperature segment. In order to calculate the retention time of an analyte for an isothermal/isocratic hold equation A 4-6 should be employed.

$$t_R = \frac{t_0}{2.3 b_T} \ln\left[e^{2.3 b_T r} \left(k_0 + 1\right) - k_0 \right] \qquad \text{(A 4-4)}$$

$$\log k_r = \log k_0 - \frac{b_T t_R}{t_0} \qquad \text{(A 4-5)}$$

$$t_R = r t_0 \left(k_0 + 1\right) \qquad \text{(A 4-6)}$$

Appendix 4-5.2 Calculation of the Retention Time of Rutin for the First Temperature Segment of the Separation shown in Figure 4-3 c

First, a number of experimental/theoretical data has to be determined and specified (Figure A 4-3).

- the column dead time t_0 (cell: G11)
- the start temperature of the temperature gradient T_{start} (cell: G12)
- the final temperature of the temperature gradient T_{final} (cell: G13)
- the change in temperature during the temperature segment (cell: G15)
- the gradient time of the temperature segment tG_1 (cell: G16)
- a cell where the temperature-gradient steepness parameter b_T will be calculated according to equation A 4-2 (cell: C12)

- a cell for the fractional migration $r_{segment1}$ of the analyte during the first temperature segment (cell: C13)
- two cells containing the values of S_T (cell: C5) and k_0 (cell: C6) which were calculated using the approaches described in Appendix 4-2 and 4-3, respectively
- a cell where the retention time will be calculated according to equation A 4-4 (cell: C15)
- a cell where the change of the retention factors during the first segment will be calculated according to equation A 4-5 (cells C16 and 17)

Figure A 4-3 shows a screenshot of the spreadsheet calculator which has been employed to calculate the retention time of rutin for the first temperature segment.

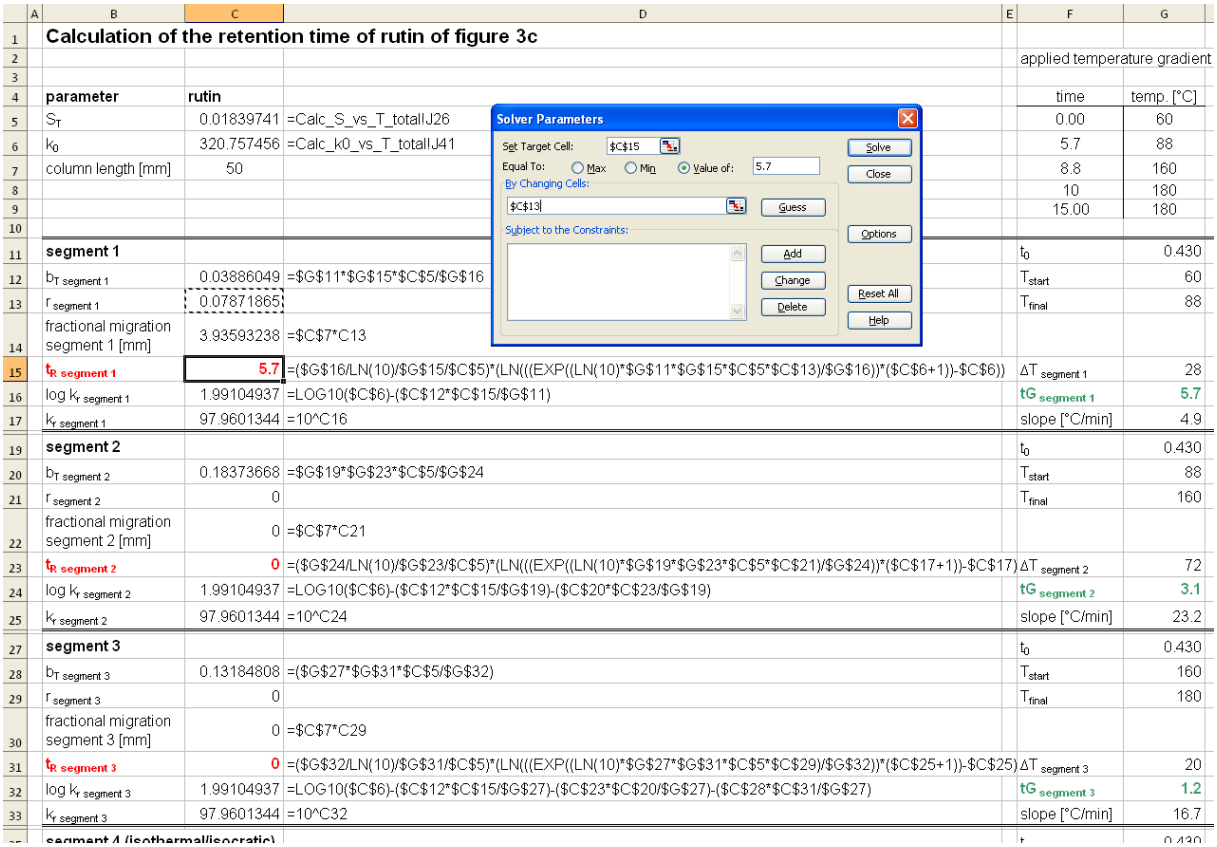
	A	B	C	D	E	F	G
1		**Calculation of the retention time of rutin of figure 3c**					
2						applied temperature gradient	
3							
4		**parameter**	**rutin**			time	temp. [°C]
5		S_T	0.01839741	=Calc_S_vs_T_totalIJ26		0.00	60
6		k_0	320.757456	=Calc_k0_vs_T_totalIJ41		5.7	88
7		column length [mm]	50			8.8	160
8						10	180
9						15.00	180
10							
11		**segment 1**				t_0	0.430
12		$b_{T\ segment\ 1}$	0.03886049	=$G$11*$G$15*$C$5/$G$16		T_{start}	60
13		$r_{segment\ 1}$	1			T_{final}	88
14		fractional migration segment 1 [mm]	50	=$C$7*C13			
15		$t_{R\ segment\ 1}$	16.5205253	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	28
16		log $k_{r\ segment\ 1}$	1.0131636	=LOG10($C$6)-($C$12*$C$15/$G$11)		$tG_{segment\ 1}$	5.7
17		$k_{r\ segment\ 1}$	10.3077433	=10^C16		slope [°C/min]	4.9
19		**segment 2**				t_0	0.430
20		$b_{T\ segment\ 2}$	0.18373668	=$G$19*$G$23*$C$5/$G$24		T_{start}	88
21		$r_{segment\ 2}$	0			T_{final}	160
22		fractional migration segment 2 [mm]	0	=$C$7*C21			
23		$t_{R\ segment\ 2}$	0	=($G$24/LN(10)/$G$23/$C$5)*(LN(((EXP((LN(10)*$G$19*$G$23*$C$5*$C$21)/$G$24))*($C$17+1))-$C$17)		$\Delta T_{segment\ 2}$	72
24		log $k_{r\ segment\ 2}$	1.0131636	=LOG10($C$6)-($C$12*$C$15/$G$19)-($C$20*$C$23/$G$19)		$tG_{segment\ 2}$	3.1
25		$k_{r\ segment\ 2}$	10.3077433	=10^C24		slope [°C/min]	23.2
27		**segment 3**				t_0	0.430
28		$b_{T\ segment\ 3}$	0.13184808	=($G$27*$G$31*$C$5/$G$32)		T_{start}	160
29		$r_{segment\ 3}$	0			T_{final}	180
30		fractional migration segment 3 [mm]	0	=$C$7*C29			
31		$t_{R\ segment\ 3}$	0	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25)		$\Delta T_{segment\ 3}$	20
32		log $k_{r\ segment\ 3}$	1.0131636	=LOG10($C$6)-($C$12*$C$15/$G$27)-($C$23*$C$20/$G$27)-($C$28*$C$31/$G$27)		$tG_{segment\ 3}$	1.2
33		$k_{r\ segment\ 3}$	10.3077433	=10^C32		slope [°C/min]	16.7
		segment 4 (isothermal/isocratic)				t_0	0.430

Figure A 4-3: Spreadsheet calculator, sheet displaying the equations that are needed to calculate the retention time and fractional migration of rutin for the first temperature segment.

The calculation starts by setting the value of the fractional migration of rutin for the first temperature segment to 1, assuming that the analyte migrates completely during the first segment. As can be seen from Figure A 4-3, this setting yields a retention time of rutin of

16.52 min (cell: C15). It has to be considered that the maximal retention time of rutin for the first segment can only be equal or lower to the temperature-gradient time of the first segment (cell: G16). Hence, the maximal retention time of rutin can only be 5.7 min or lower. In other words, if the calculated retention time of an analyte for a temperature segment is higher than the temperature-gradient time of the segment, the analyte does not elute during this temperature segment. This means that the analyte only migrates a certain distance of the total column length during the temperature segment.

Therefore, the fractional migration of the analyte has to be calculated depending on the applied temperature gradient of the first temperature segment. Figure A 4-4 shows a screenshot of the spreadsheet calculator which has been employed to calculate the fractional migration of rutin for the first temperature segment by means of MS Excel Solver.



	B	C	D	F	G
1	**Calculation of the retention time of rutin of figure 3c**				
2				applied temperature gradient	
3					
4	**parameter**	**rutin**		time	temp. [°C]
5	S_T	0.01839741	=Calc_S_vs_T_totalIJ26	0.00	60
6	k_0	320.757456	=Calc_k0_vs_T_totalIJ41	5.7	88
7	column length [mm]	50		8.8	160
8				10	180
9				15.00	180
10					
11	**segment 1**			t_0	0.430
12	$b_{T\ segment\ 1}$	0.03886049	=$G$11*$G$15*$C$5/$G$16	T_{start}	60
13	$r_{segment\ 1}$	0.07871865		T_{final}	88
14	fractional migration segment 1 [mm]	3.93593238	=$C$7*C13		
15	$t_{R\ segment\ 1}$	5.7	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))	$\Delta T_{segment\ 1}$	28
16	log $k_{r\ segment\ 1}$	1.99104937	=LOG10($C$6)-($C$12*$C$15/$G$11)	$tG_{segment\ 1}$	5.7
17	$k_{r\ segment\ 1}$	97.9601344	=10^C16	slope [°C/min]	4.9
19	**segment 2**			t_0	0.430
20	$b_{T\ segment\ 2}$	0.18373668	=$G$19*$G$23*$C$5/$G$24	T_{start}	88
21	$r_{segment\ 2}$	0		T_{final}	160
22	fractional migration segment 2 [mm]	0	=$C$7*C21		
23	$t_{R\ segment\ 2}$	0	=($G$24/LN(10)/$G$23/$C$5)*(LN(((EXP((LN(10)*$G$19*$G$23*$C$5*$C$21)/$G$24))*($C$17+1))-$C$17)	$\Delta T_{segment\ 2}$	72
24	log $k_{r\ segment\ 2}$	1.99104937	=LOG10($C$6)-($C$12*$C$15/$G$19)-($C$20*$C$23/$G$19)	$tG_{segment\ 2}$	3.1
25	$k_{r\ segment\ 2}$	97.9601344	=10^C24	slope [°C/min]	23.2
27	**segment 3**			t_0	0.430
28	$b_{T\ segment\ 3}$	0.13184808	=($G$27*$G$31*$C$5/$G$32)	T_{start}	160
29	$r_{segment\ 3}$	0		T_{final}	180
30	fractional migration segment 3 [mm]	0	=$C$7*C29		
31	$t_{R\ segment\ 3}$	0	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25)	$\Delta T_{segment\ 3}$	20
32	log $k_{r\ segment\ 3}$	1.99104937	=LOG10($C$6)-($C$12*$C$15/$G$27)-($C$23*$C$20/$G$27)-($C$28*$C$31/$G$27)	$tG_{segment\ 3}$	1.2
33	$k_{r\ segment\ 3}$	97.9601344	=10^C32	slope [°C/min]	16.7
	segment 4 (isothermal/isocratic)			t_0	0.430

Solver Parameters: Set Target Cell: $C$15; Equal To: Max / Min / Value of: 5.7; By Changing Cells: $C$13; Subject to the Constraints:; Solve; Close; Guess; Options; Add; Change; Reset All; Delete; Help

Figure A 4-4: Spreadsheet calculator, sheet displaying the equations and Solver Parameters that are needed to calculate the fractional migration of rutin for the first temperature segment.

The "Target Cell" corresponds to the retention time of rutin for the first temperature segment (cell: C15) and should be set "Equal To" a "Value of" 5.7 "By Changing Cells" that corresponds with the fractional migration (cell: C13) of rutin during the first temperature segment. Now, the solver function will search for a value of the fractional migration

($r_{segment1}$), yielding a retention time of rutin of 5.7 min. As can be seen from Figure A 4-4, the retention time of rutin was calculated to 5.7 min for the first temperature segment by changing the fractional migration to a value of approximately 0.0787 corresponding to a migration distance of rutin of 3.94 mm of the total column length during this temperature segment.

As pointed out before, because of the applied temperature gradient during the first segment, the value of the retention factor (k_0) of rutin was changed. Now, the retention factor of rutin corresponding with the final temperature of the first segment, which is equal to the start temperature of the second temperature segment, has to be calculated according to equation A 4-5 and yields a value of 97.96 (cell: C17).

Appendix 4-5.3 Calculation of the Retention Time of Rutin for the Second Temperature Segment of the Separation shown in Figure 4-3 c

Very similar to the calculation of the retention time of rutin for the first temperature segment, a number of experimental/theoretical data have to be determined and specified for the second segment (Figure A 4-5).

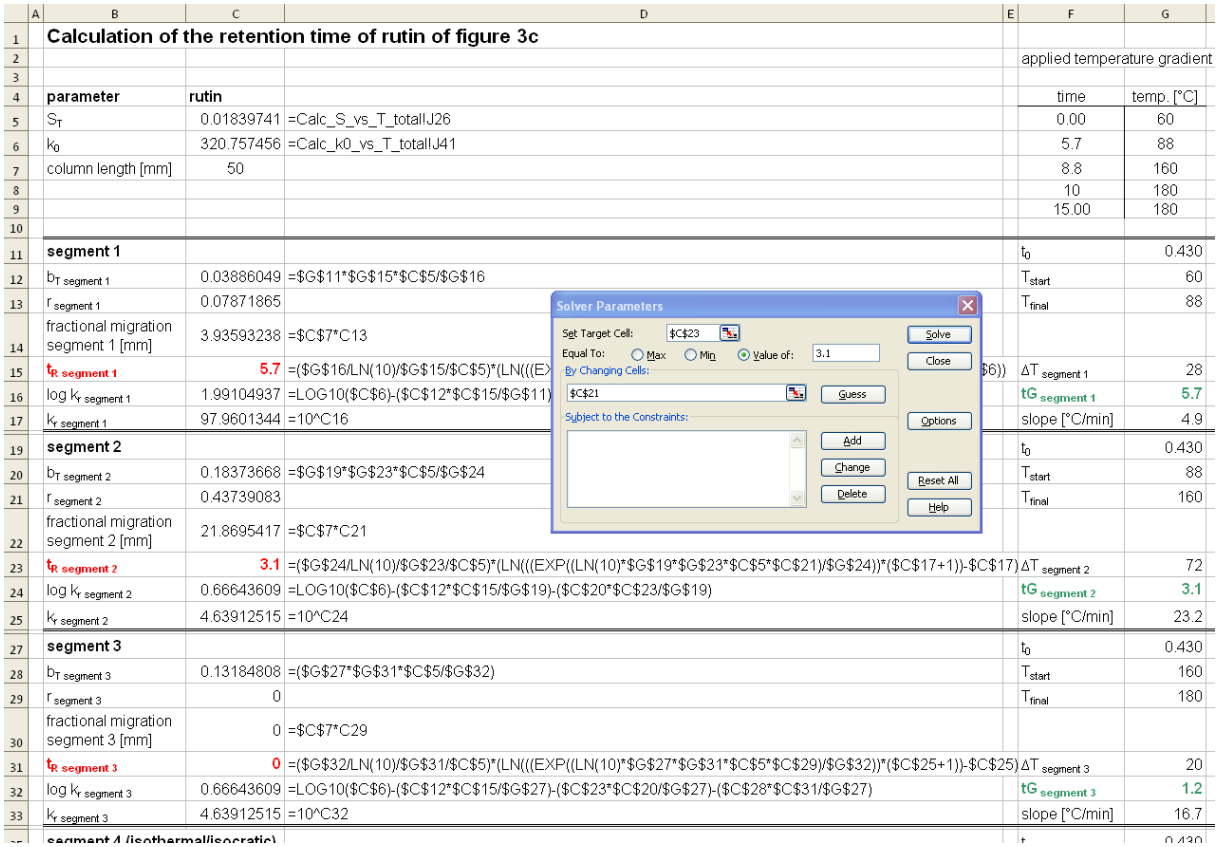
- the column dead time t_0 (cell: G19)
- the start temperature of the temperature gradient T_{start} (cell: G20)
- the final temperature of the temperature gradient T_{final} (cell: G21)
- the change in temperature during the temperature segment (cell: G23)
- the gradient time of the temperature segment tG_2 (cell: G24)
- a cell where the temperature-gradient steepness parameter b_T will be calculated according to equation A 4-2 (cell: C20)
- a cell for the fractional migration $r_{segment\,2}$ of the analyte during the second temperature segment (cell: C21)
- a cell containing the value of S_T (cell: C5)
- a cell containing the value of k_0 corresponding to the value of the retention factor calculated after the first temperature segment (cell: C17)
- a cell where the retention time will be calculated according to equation A 4-4 (cell: C23)
- a cell where the change of the retention factor during the second segment will be calculated according to equation A 4-5 (cells C24 and C25)

Figure A 4-5 shows a screenshot of the spreadsheet calculator which has been employed to calculate the retention time of rutin for the second temperature segment.

	A	B	C	D	E	F	G
1		**Calculation of the retention time of rutin of figure 3c**					
2						applied temperature gradient	
3							
4		**parameter**	**rutin**			time	temp. [°C]
5		S_T	0.01839741	=Calc_S_vs_T_totalIJ26		0.00	60
6		k_0	320.757456	=Calc_k0_vs_T_totalIJ41		5.7	88
7		column length [mm]	50			8.8	160
8						10	180
9						15.00	180
10							
11		**segment 1**				t_0	0.430
12		$b_{T\ segment\ 1}$	0.03886049	=$G$11*$G$15*$C$5/$G$16		T_{start}	60
13		$r_{segment\ 1}$	0.07871865			T_{final}	88
14		fractional migration segment 1 [mm]	3.93593238	=$C$7*C13			
15		$t_{R\ segment\ 1}$	5.7	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	28
16		log $k_{r\ segment\ 1}$	1.99104937	=LOG10($C$6)-($C$12*$C$15/$G$11)		$tG_{segment\ 1}$	5.7
17		$k_{r\ segment\ 1}$	97.9601344	=10^C16		slope [°C/min]	4.9
19		**segment 2**				t_0	0.430
20		$b_{T\ segment\ 2}$	0.18373668	=$G$19*$G$23*$C$5/$G$24		T_{start}	88
21		$r_{segment\ 2}$	0.92128135			T_{final}	160
22		fractional migration segment 2 [mm]	46.0640676	=$C$7*C21			
23		$t_{R\ segment\ 2}$	3.93816682	=($G$24/LN(10)/$G$23/$C$5)*(LN(((EXP((LN(10)*$G$19*$G$23*$C$5*$C$21)/$G$24))*($C$17+1))-$C$17)		$\Delta T_{segment\ 2}$	72
24		log $k_{r\ segment\ 2}$	0.30829193	=LOG10($C$6)-($C$12*$C$15/$G$19)-($C$20*$C$23/$G$19)		$tG_{segment\ 2}$	3.1
25		$k_{r\ segment\ 2}$	2.03372359	=10^C24		slope [°C/min]	23.2
27		**segment 3**				t_0	0.430
28		$b_{T\ segment\ 3}$	0.13184808	=($G$27*$G$31*$C$5/$G$32)		T_{start}	160
29		$r_{segment\ 3}$	0			T_{final}	180
30		fractional migration segment 3 [mm]	0	=$C$7*C29			
31		$t_{R\ segment\ 3}$	0	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25)		$\Delta T_{segment\ 3}$	20
32		log $k_{r\ segment\ 3}$	0.30829193	=LOG10($C$6)-($C$12*$C$15/$G$27)-($C$23*$C$20/$G$27)-($C$28*$C$31/$G$27)		$tG_{segment\ 3}$	1.2
33		$k_{r\ segment\ 3}$	2.03372359	=10^C32		slope [°C/min]	16.7
		segment 4 (isothermal/isocratic)				t_0	0.430

Figure A 4-5: Spreadsheet calculator, sheet displaying the equations that are needed to calculate the retention time and fractional migration of rutin for the second temperature segment.

The calculation starts with the assumption that rutin will elute completely during the second segment. Therefore, the value for the fractional migration $r_{segment\,2}$ of rutin will be set to 0.9213 ($1-r_{segment1}=0.9213$). Doing so, a retention time of rutin of 3.94 min was calculated (cell: C23). It has to be considered that the temperature-gradient time ($tG_{segment\,2}$) of the second segment was set to 3.1 min. Therefore, MS Excel Solver has to be employed to find a value for $r_{segment\,2}$ yielding a retention time for rutin of 3.1 min for the second temperature segment. Figure A 4-6 depicts the necessary equations and Solver Parameters.



	B	C	D	F	G
1	**Calculation of the retention time of rutin of figure 3c**				
2				applied temperature gradient	
3					
4	**parameter**	**rutin**		time	temp. [°C]
5	S_T	0.01839741	=Calc_S_vs_T_totalIJ26	0.00	60
6	k_0	320.757456	=Calc_k0_vs_T_totalIJ41	5.7	88
7	column length [mm]	50		8.8	160
8				10	180
9				15.00	180
10					
11	**segment 1**			t_0	0.430
12	$b_{T\ segment\ 1}$	0.03886049	=$G$11*$G$15*$C$5/$G$16	T_{start}	60
13	$r_{segment\ 1}$	0.07871865		T_{final}	88
14	fractional migration segment 1 [mm]	3.93593238	=$C$7*C13		
15	$t_{R\ segment\ 1}$	5.7	=($G$16/LN(10)/$G$15/$C$5)*(LN(((E	$\Delta T_{segment\ 1}$	28
16	log $k_{r\ segment\ 1}$	1.99104937	=LOG10($C$6)-($C$12*$C$15/$G$11)	$tG_{segment\ 1}$	5.7
17	$k_{r\ segment\ 1}$	97.9601344	=10^C16	slope [°C/min]	4.9
19	**segment 2**			t_0	0.430
20	$b_{T\ segment\ 2}$	0.18373668	=$G$19*$G$23*$C$5/$G$24	T_{start}	88
21	$r_{segment\ 2}$	0.43739083		T_{final}	160
22	fractional migration segment 2 [mm]	21.8695417	=$C$7*C21		
23	$t_{R\ segment\ 2}$	3.1	=($G$24/LN(10)/$G$23/$C$5)*(LN(((EXP((LN(10)*$G$19*$G$23*$C$5*$C$21)/$G$24))*($C$17+1))-$C$17)	$\Delta T_{segment\ 2}$	72
24	log $k_{r\ segment\ 2}$	0.66643609	=LOG10($C$6)-($C$12*$C$15/$G$19)-($C$20*$C$23/$G$19)	$tG_{segment\ 2}$	3.1
25	$k_{r\ segment\ 2}$	4.63912515	=10^C24	slope [°C/min]	23.2
27	**segment 3**			t_0	0.430
28	$b_{T\ segment\ 3}$	0.13184808	=($G$27*$G$31*$C$5/$G$32)	T_{start}	160
29	$r_{segment\ 3}$	0		T_{final}	180
30	fractional migration segment 3 [mm]	0	=$C$7*C29		
31	$t_{R\ segment\ 3}$	0	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25)	$\Delta T_{segment\ 3}$	20
32	log $k_{r\ segment\ 3}$	0.66643609	=LOG10($C$6)-($C$12*$C$15/$G$27)-($C$23*$C$20/$G$27)-($C$28*$C$31/$G$27)	$tG_{segment\ 3}$	1.2
33	$k_{r\ segment\ 3}$	4.63912515	=10^C32	slope [°C/min]	16.7
	segment 4 (isothermal/isocratic)			t_0	0.430

Figure A 4-6: Spreadsheet calculator, sheet displaying the equations and Solver Parameters that are needed to calculate the fractional migration of rutin for the second temperature segment.

The "Target Cell" corresponds to the retention time of rutin for the second temperature segment (cell: C23) and should be set "Equal To" a "Value of" 3.1 "By Changing Cell" that corresponds to the fractional migration (cell: C21) of rutin during the second segment. Afterwards, the solver function will search for a value of $r_{segment\,2}$ yielding a retention time of 3.1 min for rutin. A value of approximately 0.4374 gives the desired retention time and corresponds to a migration distance of rutin of 21.87 mm of the total column length during the second temperature segment.

Finally, the retention factor (k_0) of rutin corresponding to the final temperature of the second segment, which is equal to the start temperature of the third temperature segment, has to be calculated according to equation A 4-5 and yields a value of 4.64 (cell: C25).

Appendix 4-5.4 Calculation of the Retention Time of Rutin for the Third Temperature Segment of the Separation shown in Figure 4-3 c

For the calculation of the retention of rutin for the third temperature segment, a number of experimental/theoretical data has to be determined and specified (Figure A 4-7).

- the column dead time t_0 (cell: G27)
- the start temperature of the temperature gradient T_{start} (cell: G28)
- the final temperature of the temperature gradient T_{final} (cell: G29)
- the change in temperature during the temperature segment (cell: G31)
- the gradient time of the temperature segment tG_3 (cell: G32)
- a cell where the temperature-gradient steepness parameter b_T will be calculated according to equation A 4-2 (cell: C28)
- a cell for the fractional migration $r_{segment3}$ of the analyte during the third temperature segment (cell: C29)
- a cell containing the value of S_T (cell: C5)
- a cell containing the value of k_0 corresponding to the value of the retention factor calculated after the second temperature segment (cell: C25)
- a cell where the retention time will be calculated according to equation A 4-4 (cell: C31)
- a cell where the change of the retention factor during the third segment will be calculated according to equation A 4-5 (cells C32 and C33)

Figure A 4-7 depicts a screenshot of the spreadsheet calculator which has been employed to calculate the retention time of rutin for the third temperature segment.

The calculation starts again with the assumption that rutin will elute during the third segment of the gradient by setting the value of the fractional migration $r_{segment3}$ to 0.4839 ($1 - r_{segment1} - r_{segment2} = 0.4839$), yielding a retention time of 0.90 min (cell: C31) for rutin for the third temperature segment. As can be seen, this value is smaller than the temperature-

gradient time of 1.2 min (cell: G32) and points out that rutin elutes during the third segment from the column.

Finally, the retention times of rutin during each segment sum up to 9.70 min (cell: C44), which represents the total retention time of rutin.

	A	B	C	D	E	F	G
13		$r_{segment\ 1}$	0.07871865			T_{final}	88
14		fractional migration segment 1 [mm]	3.93593238	=$C$7*C13			
15		$t_{R\ segment\ 1}$	5.7	=($G$16/LN(10)/$G$15/$C$5)*(LN(((EXP((LN(10)*$G$11*$G$15*$C$5*$C$13)/$G$16))*($C$6+1))-$C$6))		$\Delta T_{segment\ 1}$	28
16		log $k_{r\ segment\ 1}$	1.99104937	=LOG10($C$6)-($C$12*$C$15/$G$11)		$tG_{segment\ 1}$	5.7
17		$k_{r\ segment\ 1}$	97.9601344	=10^C16		slope [°C/min]	4.9
19		**segment 2**				t_0	0.430
20		$b_{T\ segment\ 2}$	0.18373668	=$G$19*$G$23*$C$5/$G$24		T_{start}	88
21		$r_{segment\ 2}$	0.43739083			T_{final}	160
22		fractional migration segment 2 [mm]	21.8695417	=$C$7*C21			
23		$t_{R\ segment\ 2}$	3.1	=($G$24/LN(10)/$G$23/$C$5)*(LN(((EXP((LN(10)*$G$19*$G$23*$C$5*$C$21)/$G$24))*($C$17+1))-$C$17)		$\Delta T_{segment\ 2}$	72
24		log $k_{r\ segment\ 2}$	0.66643609	=LOG10($C$6)-($C$12*$C$15/$G$19)-($C$20*$C$23/$G$19)		$tG_{segment\ 2}$	3.1
25		$k_{r\ segment\ 2}$	4.63912515	=10^C24		slope [°C/min]	23.2
27		**segment 3**				t_0	0.430
28		$b_{T\ segment\ 3}$	0.13184808	=($G$27*$G$31*$C$5/$G$32)		T_{start}	160
29		$r_{segment\ 3}$	0.48389052			T_{final}	180
30		fractional migration segment 3 [mm]	24.1945259	=$C$7*C29			
31		$t_{R\ segment\ 3}$	0.90339849	=($G$32/LN(10)/$G$31/$C$5)*(LN(((EXP((LN(10)*$G$27*$G$31*$C$5*$C$29)/$G$32))*($C$25+1))-$C$25)		$\Delta T_{segment\ 3}$	20
32		log $k_{r\ segment\ 3}$	0.38943293	=LOG10($C$6)-($C$12*$C$15/$G$27)-($C$23*$C$20/$G$27)-($C$28*$C$31/$G$27)		$tG_{segment\ 3}$	1.2
33		$k_{r\ segment\ 3}$	2.45150583	=10^C32		slope [°C/min]	16.7
35		**segment 4 (isothermal/isocratic)**				t_0	0.430
36		$b_{T\ segment\ 4}$	0	=($G$35*$G$39*$C$5/$G$40)		T_{start}	180
37		$r_{segment\ 4}$	0			T_{final}	180
38		fractional migration segment 1 [mm]	0	=$C$7*C37			
39		$t_{R\ segment\ 4}$	0	=$G$35*$C$37*($C$33+1)		$\Delta T_{segment\ 4}$	0
40		log $k_{r\ segment\ 4}$	0.38943293	=C32		$tG_{segment\ 4}$	5
41		$k_{r\ segment\ 4}$	2.45150583	=10^C40		slope [°C/min]	0.0
43		sum fractional migration [mm]	50	=C14+C22+C30+C38			
44		$t_{R\ total}$	**9.70**	=C39+C31+C23+C15			
45							

Figure A 4-7: Spreadsheet calculator, sheet displaying the equations that are needed to calculate the retention time and fractional migration of rutin for the third temperature segment.

Appendix 4-6 Robustness of a Temperature-Gradient Method for the Separation of Selected Food Additives

In order to underline that small changes of the temperatures of the gradient points will also have an effect of the retention times of the food additives, Figure A 4-8 shows the resulting chromatograms, when the temperature-gradient points of the separation method were changed by ± 1 °C (see also section 4.4.4).

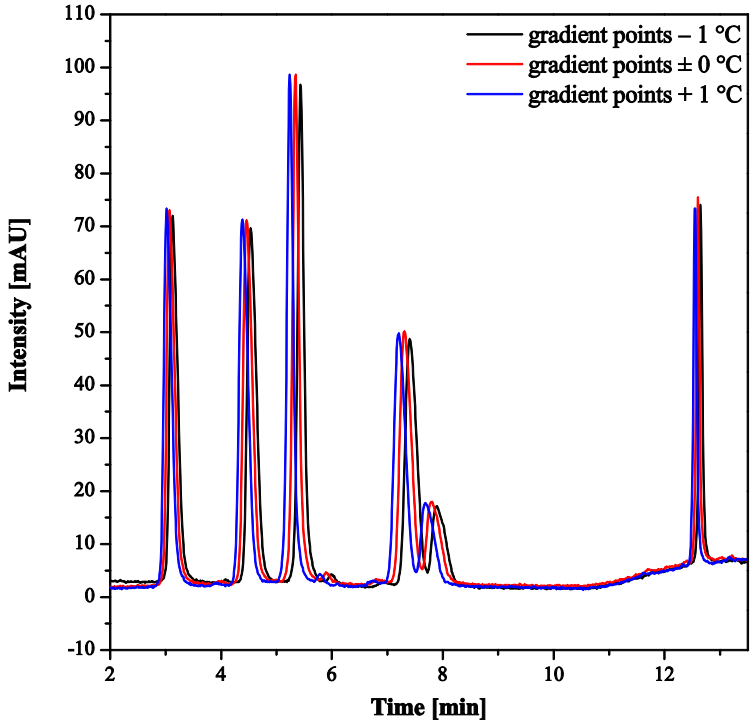


Figure A 4-8: Overlay of three chromatograms of the separation of six food additives. The temperature-gradient points were changed ± 1 °C. Chromatographic conditions: Waters XBridge C_{18} (50 × 3.0 mm, 3.5 µm); mobile phase: water + 0.1% formic acid; flow rate: 0.5 mL min^{-1}; injection volume: 1 µL; temperature gradient (± 0 °C): 0 min at 50 °C; 10.37 min at 128 °C; 12.00 at 180 °C; 14.00 min at 180 °C.

As can be seen, if the temperature of the gradient points were decreased about 1 °C, an increase of the retention times of the food additives was observed. In contrast, if the temperature of the gradient points were increased about 1 °C, a decrease of the retention times of the food additives was observed. This means that small changes of the temperature of the temperature-gradient points will have a significant effect on the repeatability as well as the robustness of a temperature-gradient method. In other words, during the validation of a temperature-gradient method, the user should carefully check how the separation method will be affected by small changes of the temperature as well as the time of the gradient points.

List of Symbols of Appendix for Chapter 4

b_T	temperature-gradient steepness parameter
k_0	retention factor of an analyte i at the start of the temperature gradient
r	fractional migration of an analyte i during a segment of the temperature-gradient method
S_T	analyte specific constant of the LES retention model
ΔT	difference between start and final temperature of the temperature gradient
t_0	column dead time
T_{final}	final temperature of the temperature gradient
tG	temperature-gradient time
t_R	retention time of an analyte i
T_{start}	start temperature of the temperature gradient

Chapter 5. A General Strategy for Performing Temperature Programming in High Performance Liquid Chromatography – Prediction of Simultaneous Solvent and Temperature Gradients

5.1 Introduction

In high performance liquid chromatography (HPLC) the parameter temperature is often discussed only in terms of speeding up a separation [1,2]. This is due to the fact that increasing the temperature lowers the viscosity of the mobile phase and a reduced backpressure is observed. Hence, the flow rate can be increased concurrently resulting in a decrease of the analysis time. An increase of temperature yields also a flattened van Deemter curve. Thus, the increased flow rate only slightly affects the efficiency [3,4]. In addition, the reduced backpressure enables the use of smaller particles or longer columns which increases the efficiency significantly [5].

However, temperature is not only a tool to speed up a separation or to increase efficiency; temperature also plays an important role to change selectivity in liquid chromatography, especially for polar and ionizable compounds [6,7]. Chen and Horvath compared solvent gradient elution and temperature programming for the separation of selected alkylbenzenes and proteins [8]. They concluded that temperature programming can only be an insufficient alternative to solvent gradient elution. However, they also pointed out that temperature programming in combination with solvent gradient elution could be employed as fine tuning to enhance the critical resolution of structurally similar macromolecules such as proteins. Another impressive example was given by Vanhoenacker and Sandra [3] who developed a method for the separation of 20 pesticides by a combined solvent and temperature gradient. They could show that an isothermal baseline separation within a temperature range from 40 °C to 90 °C by means of a solvent gradient was not possible, and a combination of simultaneous solvent and temperature programming was required for a baseline separation. A similar example was given by Giegold et al. [9] who could also show that a baseline separation of eight sulfonamides and trimethoprim was only possible in dual gradient mode where a solvent and temperature gradient were applied simultaneously. However, it has to be considered that method development based on temperature programming of the examples cited above was governed by trial and error. Although the parameter temperature is considered during method development in LC and several commercially available method development software packages such as ChromSwordAuto [10] or DryLab [11] have implemented retention models where temperature is considered, these models are restricted to isothermal operation mode. This problem was first considered by Nikitas and Pappa-Louisi. They developed prediction models which permit simulation of retention times when solvent composition and temperature are changed simultaneously [12,13]. Up to now, their models

were tested using only linear temperature gradients within a relatively small temperature interval from 15 °C to 75 °C where only moderate gradient slopes from 2 °C min^{-1} up to 10 °C min^{-1} were applied. Recently, Cela and co-workers have described computer-assisted method development in high-temperature liquid chromatography based on an evolutionary algorithm [14]. The developed approach also permits dual mode simulations of retention times when solvent composition and temperature are changed simultaneously. In 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 [14]. However, based on the data given by Cela and co-workers we calculated major relative errors up to 10% for simultaneous solvent and temperature-gradient predictions.

Lately, we could show that the linear elution strength model from temperature-programmed gas chromatography (GC) can be employed for predictions of simple linear as well as more complex segmented temperature gradients in temperature-programmed liquid chromatography [15,16]. It was shown that temperature gradients with slopes up to 30 °C min^{-1} in a temperature interval from 50 °C up to 180 °C can be predicted with high accuracy. On the basis of these findings and the knowledge of the similarity of the linear elution strength (LES) and the linear solvent strength (LSS) retention models, the aim of the present study was to develop a combined model in order to predict simultaneous solvent and temperature gradients in LC.

5.2 Experimental Section

5.2.1 Chemicals

High-purity deionized water was prepared by an Elix 10 – Milli-Q Plus water purification system (Millipore, Eschborn, Germany). Acetonitrile (Optigrade), acetone (Optigrade) and methanol (Optigrade) were purchased from LGC Standards (Wesel, Germany). In this work two different analyte mixtures were used. The first one was a commercially available mixture of thirteen aldehyde-2,4-dinitrophenylhydrazones (aldehyde-2,4-DNPH) and ketone-2,4-dinitrophenylhydrazones (ketone-2,4-DNPH). The substances and the concentration in the mixture are given in Table 5-1. This mixture is called A-DNPH mixture during this work. For the measurements, this mixture was diluted with water by a factor of three and then used as injection solution.

Table 5-1: Substances of the A-DNPH test mixture.

analyte	CAS number	elution order	conc. standard [µg mL^{-1}]	conc. injected solution [µg mL^{-1}]
formaldehyde-2,4-DNPH	1081-15-8	1	210.0	70.0
acetaldehyde-2,4-DNPH	1019-57-4	2	152.0	50.7
acrolein-2,4-DNPH	888-54-0	3	126.4	42.1
acetone-2,4-DNPH	1567-89-1	4	124.2	41.4
propionaldehyde-2,4-DNPH	725-00-8	5	124.2	41.4
crotonaldehyde-2,4-DNPH	1527-96-4	6	108.2	36.1
methacrolein-2,4-DNPH	5077-73-6	7	108.1	36.0
2-butanone-2,4-DNPH	958-60-1	8	106.0	35.3
butyraldehyde-2,4-DNPH	1527-98-6	9	106.4	35.5
benzaldehyde-2,4-DNPH	1157-84-2	10	82.0	27.3
valeraldehyde-2,4-DNPH	2057-84-3	11	92.0	30.7
m-tolualdehyde 2,4-DNPH	2880-05-9	12	76.0	25.3
hexaldehyde-2,4-DNPH	1527-97-5	13	84.0	28.0

The second one was a mixture of eight polycyclic aromatic hydrocarbons (PAHs) which consisted of naphthalene, acenaphthylene, fluorene, anthracene, pyrene, chrysene, benzo(k)fluoranthene, and indeno(1,2,3,-cd)pyrene. Stock solutions were prepared by dissolving an equivalent amount of each PAH in a mixture of 50/50 (v/v) deionized water/acetonitrile to obtain a concentration of 0.5 mg mL^{-1}. For the measurements, a mixture was prepared by adding an equivalent amount of each stock solution to obtain a concentration

of 0.0625 mg mL^{-1} of each analyte in the mixture. All chemicals employed in this study except for the solvents were purchased from Sigma-Aldrich (Seelze, Germany) and of p. a. grade.

5.2.2 HPLC System

Two different HPLC systems (Agilent 1200 Rapid Resolution and Shimadzu LC 10) were used to collect the chromatographic data. The Agilent 1200 Series RRLC (Agilent Technologies, Waldbronn, Germany) consisted of a G1312B SL binary pump, a G1379B degasser, a G1367C autosampler and a G1315C diode array detector (DAD). To heat the mobile and stationary phase an SIM HT-HPLC 200 high-temperature column oven (SIM, Scientific Instruments Manufacturer, Oberhausen, Germany) was used [17,18]. The Shimadzu LC 10 (Shimadzu, Duisburg, Germany) consisted of two LC-10AD_{VP} pumps, a DGU-14 A degasser, an SIL-10AD_{VP} autosampler, an SPD-M10A_{VP} diode array detector (DAD), an SCL-10A_{VP} controller, and the SIM HT-HPLC 200 high-temperature column oven. 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, Agilent ChemStation for LC 3D systems (Rev. B.04.02 [96]) and Shimadzu LCsolution (version 1.21 SP 1) were employed.

5.2.3 Simultaneous Solvent and Temperature-Gradient Measurements of the A-DNPH Mixture

The measurements of the A-DNPH mixture were performed on the Agilent HPLC system using an Agilent Zorbax StableBond C_{18} column (50 × 3.0 m, 1.8 µm). As organic solvents (% B) in the mobile phase acetone and methanol were employed. The flow rate was set to 1.0 mL min^{-1} and the injection volume was set to 5 µL. UV detection was performed at a wavelength of 360 nm. At first, linear solvent gradients from 5% to 100% B in 5, 10 and 30 minutes were carried out under isothermal conditions at 70 °C. Afterwards, the same solvent gradient measurements were applied and concurrently overlaid by linear temperature gradients from 70 °C to 120 °C (ΔT = 50 °C) within 5, 10, and 30 minutes.

5.2.4 Simultaneous Solvent and Temperature-Gradient Measurements of the PAH Mixture

The measurements of the PAH mixture were performed on the Shimadzu HPLC system using a Waters XBridge C_{18} column (75 × 4.6 m, 2.5 µm). As mobile phase a mixture of deionized water and methanol (% B) at a flow rate of 1.0 mL min^{-1} was employed. The injection volume was set to 3 µL and UV detection was carried out at a wavelength of 254 nm. First, linear solvent gradients from 40% to 100% methanol in 30, 60, and 90 minutes were carried out at a temperature of 50 °C. Afterwards, the same solvent gradient measurements were applied and concurrently overlaid by linear temperature gradients from 50 °C to 180 °C (ΔT = 130 °C) within 30, 60, and 90 minutes.

5.3 Theory

In order to perform systematic method development in liquid chromatography, the so called linear solvent strength model (LSS), which was introduced by Snyder and co-workers can be used [19,20]. On the basis of two experimental solvent gradient measurements it is possible to predict the retention time t_R of an analyte with high accuracy (equations 5-1 and 5-2).

$$t_R = \frac{t_0}{b_\phi}\log\left(2.303 k_{0\phi} b_\phi + 1\right) + t_0 + t_D \tag{5-1}$$

$$\text{with } b_\phi = \frac{t_0 \Delta\phi S_\phi}{t_G} \tag{5-2}$$

Here, t_0 is the column dead time, $k_{0\phi}$ is the value of the retention factor at the start of the solvent gradient at constant temperature and t_D is the dwell time of the gradient system. The solvent gradient-steepness parameter b_ϕ consists of the solute constant S_ϕ, the change in the volume fraction of the organic solvent during the gradient $\Delta\phi$ ($\Delta\phi = \phi_{final} - \phi_{start}$) and the solvent gradient time t_G. As mentioned before, using two initial runs, values of b_ϕ or rather S_ϕ and $k_{0\phi}$ for each analyte are derived by a numerical solution of equations 5-1 and 5-2 using the Microsoft Excel Solver [19-21].

In order to predict the retention time of an analyte depending on a temperature gradient in liquid chromatography we have shown that the linear elution strength model (LES) from temperature-programmed gas chromatography could successfully be adapted to temperature-

programmed LC [15,16]. Using the LES model the retention time t_R of an analyte can be simulated as a function of experimental conditions using equations 5-3 and 5-4:

$$t_R = \frac{t_0}{b_T \ln(10)} \ln\left[e^{\ln(10) b_T} \left(k_{0T} + 1\right) - k_{0T} \right] \tag{5-3}$$

$$\text{with } b_T = \frac{t_0 S_T \Delta T}{T_G} \tag{5-4}$$

where t_0 is the column dead time and k_{0T} is the value of the retention factor of the solute at the start of the temperature gradient under isocratic 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 T_G. The required model parameters b_T or rather S_T and k_{0T} can then be calculated by a numerical solution of equations 5-3 and 5-4 using the Microsoft Excel Solver. This procedure is very similar to the numerical solution of the LSS relationship [21-23]. Snyder and co-workers [24-27] could show that the parameter temperature has a pronounced effect on the retention factor of an analyte in isothermal operation mode. If the temperature is increased in reversed phase liquid chromatography (RP-LC), the retention factor decreases. The decrease of the retention factor is also expected in temperature-programming mode. In other words, the observed retention factor of an analyte for a linear solvent gradient at constant temperature like 70 °C should decrease if this solvent gradient is concurrently overlaid by a temperature gradient from e.g. 70 °C to 120 °C. The difference between the observed retention factors will be attributed to the influence of the overlaid temperature gradient. In order to combine the LSS and LES model to predict simultaneous solvent and temperature gradients, at least four input measurements are required. Two solvent gradient runs which differ in the slope of the gradient at constant temperature (isothermal) and the same two solvent gradient runs which are overlaid by temperature gradients which also differ in the slope of the temperature gradient. Moreover, the start temperature of the temperature gradients must be equal to the temperature of the isothermal solvent gradient measurements. Subsequently, the two isothermal solvent gradient measurements can be employed to calculate the required LSS model parameters b_ϕ or rather S_ϕ and $k_{0\phi}$ for each analyte by a numerical solution of equations 5-1 and 5-2. In order to calculate the required LES model parameters S_T and k_{0T}, the difference between the retention times of the isothermal solvent gradient measurements and the measurements which are overlaid by temperature gradients have to be employed.

5.4 Results and Discussion

At first, to clarify the influence of temperature on retention in liquid chromatography Figure 5-1 depicts four chromatograms of the separation of the A-DNPH mixture using methanol (Figure 5-1 a, c) and acetone (Figure 5-1 b, d) as organic co-solvent in the mobile phase. If a linear solvent gradient from 5% to 100% methanol in 30 minutes is applied at 70 °C the last compound elutes after approximately 19.7 minutes (Figure 5-1 a). In the case of the same solvent gradient overlaid by a linear temperature gradient from 70 °C to 120 °C within 30 minutes, a retention time of 17.6 minutes was observed for the last eluting compound (Figure 5-1 c).

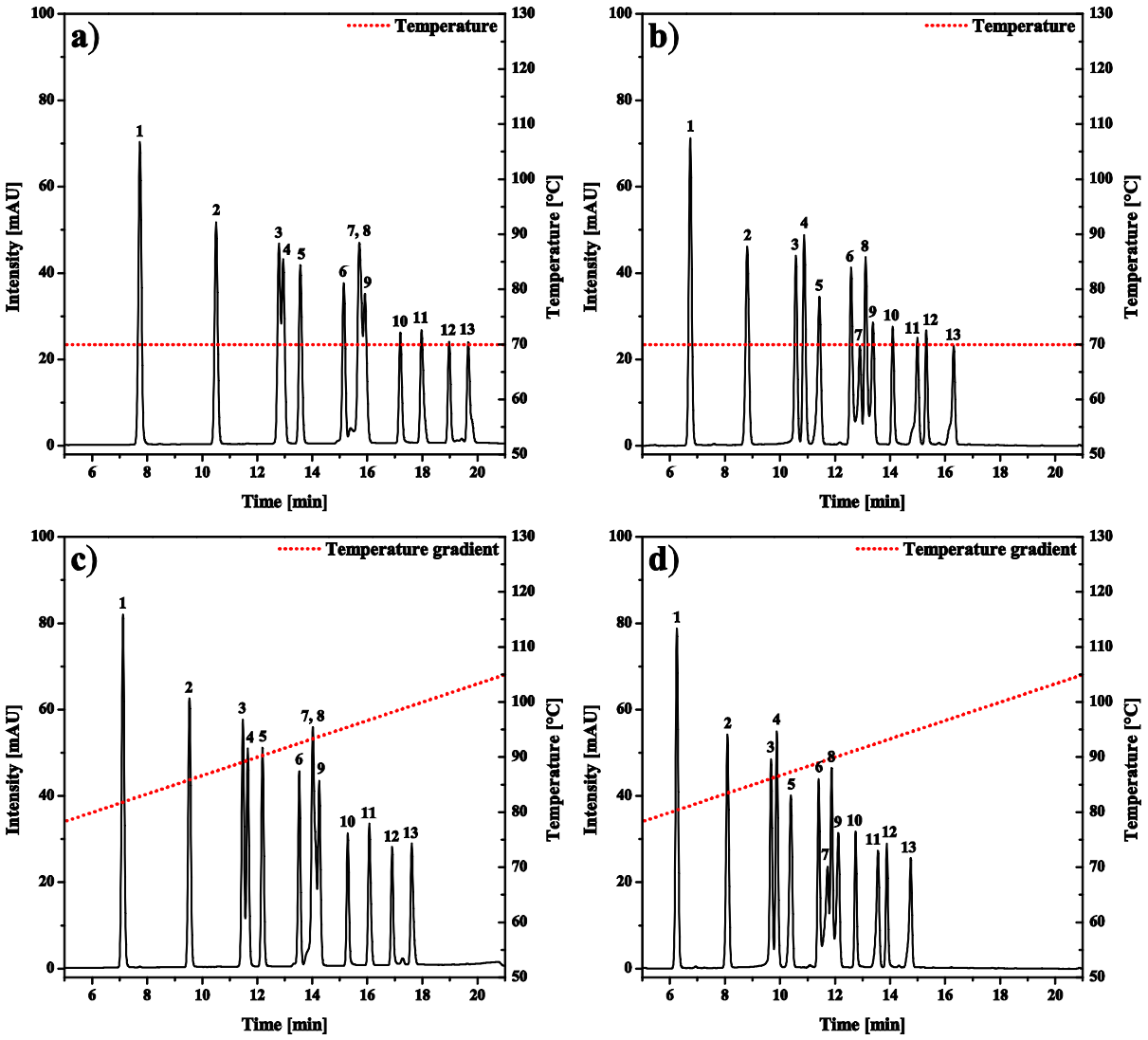


Figure 5-1: Separation of selected A-DNPHs on an Agilent Zorbax StableBond C_{18} column. Chromatographic conditions: mobile phase: A: deionized water, B: acetone (a, c), methanol (b, d); solvent gradient: 5% - 100% B in 30 minutes; temperature: isothermal at 70 °C (a, b), temperature gradient from 70 °C-120 °C in 30 minutes (c, d); injection volume: 5 µL; detection: UV at 360 nm. For analytes and elution order see Table 5-1.

Similar results were obtained using acetone as organic co-solvent in the mobile phase. The retention times of selected analytes decrease when a solvent gradient was concurrently overlaid by a temperature gradient (Figure 5-1 b and d). This can also be seen from Table 5-2 where the retention factors of selected A-DNPHs under isothermal and temperature-gradient conditions are compared.

Table 5-2: Comparison of retention factors of selected A-DNPHs using simultaneous variation of solvent composition and temperature ($k_{e,\phi,T}$) vs isothermal solvent gradient measurements ($k_{e,\phi}$).

analyte	methanol		acetone		methanol		acetone	
	$k_{e,\phi}$	$k_{e,\phi,T}$	$k_{e,\phi}$	$k_{e,\phi,T}$	difference of k	difference of k [%]	difference of k	difference of k [%]
1	34.14	31.35	29.60	27.38	2.21	8.2	2.21	7.5
2	46.71	42.31	38.99	35.75	3.25	9.4	3.25	8.3
3	57.10	51.13	47.05	42.93	4.12	10.5	4.12	8.8
4	57.80	51.95	48.41	43.90	4.51	10.1	4.51	9.3
5	60.62	54.37	50.93	46.20	4.73	10.3	4.73	9.3
6	67.80	60.44	56.14	50.81	5.33	10.9	5.33	9.5
7	70.38	62.71	57.63	52.26	5.36	10.9	5.36	9.3
8	70.38	63.74	58.57	52.93	5.64	9.4	5.64	9.6
9	71.29	63.74	59.78	54.05	5.73	10.6	5.73	9.6
10	77.14	68.46	63.02	56.90	6.12	11.2	6.12	9.7
11	80.65	72.04	67.11	60.60	6.52	10.7	6.52	9.7
12	85.20	75.80	68.60	62.08	6.51	11.0	6.51	9.5
13	88.35	79.04	73.11	66.02	7.10	10.5	7.10	9.7

It becomes apparent that the retention factors of all analytes decrease by approximately 10% when the solvent gradient measurements are overlaid by temperature gradients (ΔT = 50 °C). This is true for both data sets using methanol as well as acetone as organic solvents in the mobile phase.

Furthermore, temperature also affects the selectivity of an RP-LC separation which can also be seen in Figure 5-1. In the case of an isothermal solvent gradient elution using methanol as organic modifier (Figure 5-1 a) there are two groups of peaks which are insufficiently separated (Peak group 3/4 and group 7/8/9). If the solvent gradient is overlaid by a temperature gradient (Figure 5-1 c), the resolution between peaks 3/4 is increased from 0.65 to 1.01. Similar to peak pair 3/4 also the resolution between double peak 7/8 and peak 9 is

increased from 0.69 to 1.04. In contrast, if acetone is used as organic solvent in the mobile phase, the resolution between these peaks decreases as the solvent gradient is overlaid by a temperature gradient (Figure 5-1 b and Figure 5-1 d). For example, the resolution between peaks 3/4 decreases from 1.63 to 1.30 and the resolution between peak pair 7/8 and peak 9 decreases from 1.03 to 0.74. These results underline that the parameter temperature and temperature gradients can be employed as fine tuning tool to improve the separation in liquid chromatography. Moreover, the results shown in Figure 5-1 point out that the organic modifier can also have a pronounced effect on the selectivity of the separation. The resolution between peaks 3/4 and peaks 7/8/9 is significantly higher using acetone as organic solvent when compared to the separation where methanol is used under isothermal conditions (Figure 5-1 a, b). From a practical point of view, it would be advantageous to perform a solvent screening to find out the optimal organic modifier before a change of the HPLC column is considered [28].

In order to predict retention times of selected A-DNPHs for simultaneous solvent and temperature gradients the approach described in the theoretical section was applied. As input data two solvent gradient measurements from 5% to 100% methanol within 10 and 30 minutes at 70 °C and the same solvent gradient runs which are overlaid by temperature gradients from 70 °C to 120 °C also within 10 and 30 minutes were employed. The two isothermal solvent gradient measurements were then used to calculate the necessary LSS parameters S_ϕ and $k_{0\phi}$ for each analyte. Afterwards, the required LES parameters S_T and k_{0T} were calculated. Unfortunately, the data fitting process was not successful because the effect on the target parameter was too small to solve the system of equations. Similar results were observed during data fitting using the data of acetone. Although the required LSS parameters could be calculated successfully based on the isothermal solvent gradient input measurements, it was not possible to solve the equations for the LES relationship. We suppose that this problem is related to the minor influence of temperature on retention. That means data fitting would be only successful when experimental conditions had been chosen where the influence of temperature is more pronounced on the retention of the analytes. To test this hypothesis selected PAHs were employed as analytes. The investigated temperature interval was enlarged from 50 °C to 180 °C (ΔT = 130 °C). Because of the much higher end temperature a Waters XBridge C_{18} column was chosen for these experiments [29]. Moreover, the slopes of the employed solvent gradients were decreased from 9.5% B min^{-1} and 3.17% B min^{-1} to 2.0% B min^{-1} and 0.67% B min^{-1}, respectively. Figure 5-2 shows two of

these chromatograms under isothermal (Figure 5-2 a) and temperature-gradient conditions (Figure 5-2 b). In addition, Table 5-3 compares the observed retention factors of selected PAHs which are represented in Figure 5-2. It can be seen that the retention time of the last eluting compound decreases from 24.8 minutes to 17.6 minutes if the isothermal solvent gradient (Figure 5-2 a) is overlaid by a temperature gradient (Figure 5-2 b).

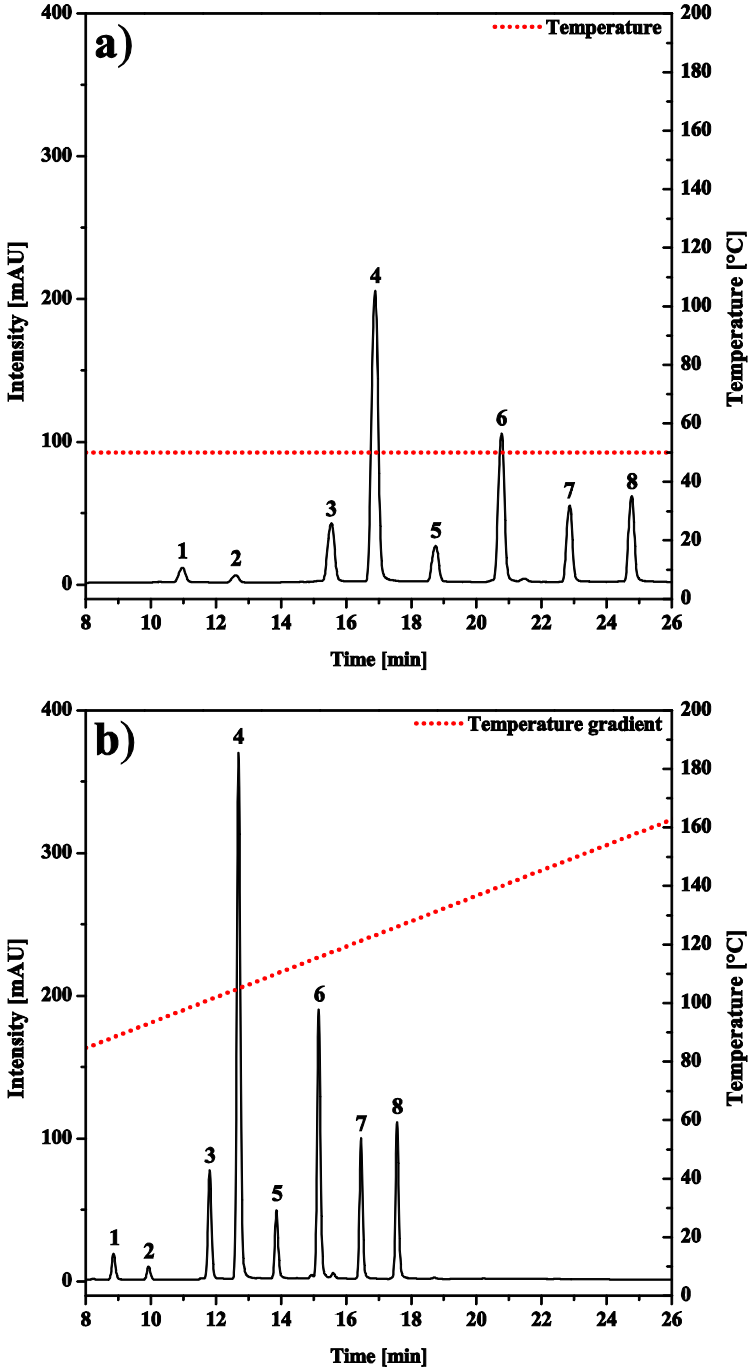


Figure 5-2: Separation of selected PAHs on a Waters XBridge C_{18} column. Chromatographic conditions: mobile phase: A: deionized water, B: methanol; solvent gradient: 40% - 100% B in 30 minutes; temperature: a) isothermal at 50 °C, b) temperature gradient: 50 °C - 180 °C in 30 minutes; injection volume: 3 µL; detection: UV at 254 nm. For analytes and elution order see Table 5-3.

In Table 5-3 it is apparent that the influence of the temperature gradient on the retention factors (of selected PAHs) is more pronounced when compared to the measurements of the A-DNPH mixture. Here, the overlaid temperature gradient yields a decrease of the retention factor of the PAHs between 20% and 30%.

Table 5-3: Comparison of retention factors of selected PAHs using simultaneous variation of solvent composition and temperature ($k_{e,\phi,T}$) vs isothermal solvent gradient measurements ($k_{e,\phi}$).

analyte	**elution order**	$k_{e,\phi}$	$k_{e,\phi,T}$	**difference of k**	**difference of k [%]**
naphthalene	1	13.24	10.48	2.76	20.8
acenaphthylene	2	15.36	11.87	3.49	22.7
fluorene	3	19.18	14.32	4.86	25.3
anthracene	4	20.93	15.47	5.46	26.1
pyrene	5	23.35	16.98	6.37	27.3
chrysene	6	26.00	18.67	7.33	28.2
benzo(k)fluoranthene	7	28.71	20.35	8.37	29.1
indeno(1,2,3,-cd)pyrene	8	31.19	21.79	9.41	30.2

With respect to prediction of retention times of selected PAHs for simultaneous solvent and temperature gradients, the approach described in the theoretical section was also employed. As input data two solvent gradient measurements from 40% to 100% methanol within 30 and 90 minutes at 50 °C and the same solvent gradients which are overlaid by temperature gradients from 50 °C to 180 °C also within 30 and 90 minutes were used. In this case data fitting of the LSS and LES model was successful, and four combined solvent and temperature gradients were predicted. Figure 5-3 presents a comparison of predicted vs experimental retention times of each PAH. The solid line in Figure 5-3 shows ideal prediction, i.e., $y = x$. Note that the data points which are shown in Figure 5-3 were not included in the data fitting process. The results indicate that the predicted retention times match the experimental retention times very well, which is also underlined by the relative error. The relative error ranges between 0.1% and 2.1%, and an average relative error of 0.6% was calculated.

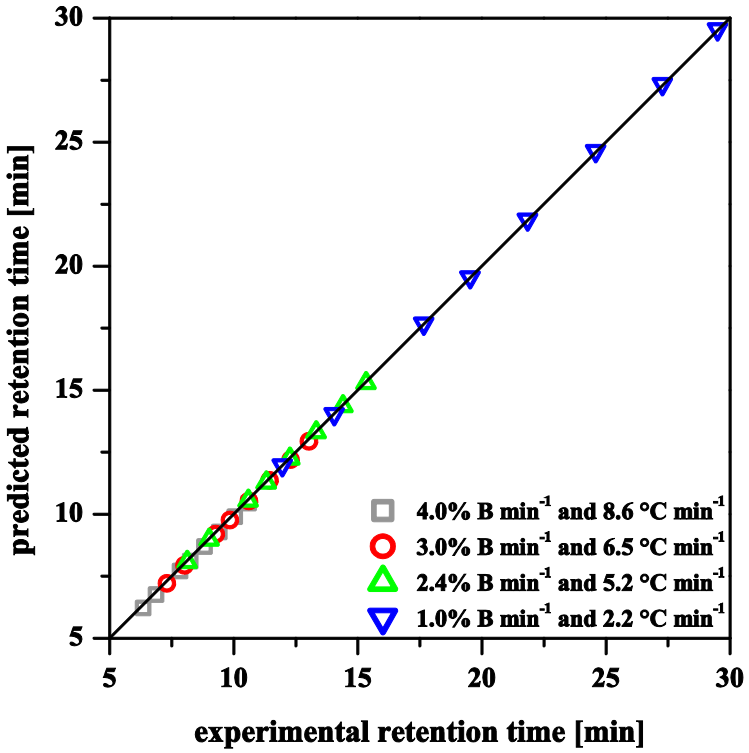


Figure 5-3: Comparison of predicted retention times calculated by a combination of the LSS and LES model vs experimental retention times of selected PAHs. Predictions based on four experimental measurements, two solvent gradients (0.67% B min^{-1} and 2% B min^{-1}) at 70 °C and the same solvent gradients which are overlaid by temperature gradients (1.4 °C min^{-1} and 4.3 °C min^{-1}).

The accuracy of retention time predictions based on the described combination of the LSS and LES model are comparable with findings by Nikitas and co-workers [12]. They reported a maximal relative error of 3.4% and an average error of 1.1% of predicted retention times for simultaneous solvent and temperature programming. During their study a temperature interval from 15 °C to 75 °C (ΔT = 60 °C) was investigated using temperature gradients with slopes from 2 °C min^{-1} to 10 °C min^{-1}. These temperature gradients were concurrently overlaid by solvent gradients from 30% to 70% acetonitrile (ACN) with slopes from 0.6% ACN min^{-1} to 1.5% ACN min^{-1}. The measurements were performed on a normal-bore HPLC column (150 × 4.6 mm, 3.5 µm) using a conventional air-bath HPLC column oven. The group of Cela and co-workers also introduced a methodology which permits retention time predictions for simultaneous solvent and temperature programming in liquid chromatography [14]. On the basis of the data given by Cela and co-workers we calculated major relative errors of predicted retention times of up to 10%. During their study a large temperature interval from 40 °C to 180 °C (ΔT = 140 °C) was investigated using gradient slopes up to 20 °C min^{-1}. The temperature gradients were overlaid by solvent gradients between 5% and 95% methanol

(MeOH) where gradient slopes from 2.5% MeOH min^{-1} to 15% MeOH min^{-1} were applied. Also in their study a normal-bore HPLC column (50 × 4.6 mm, 5 µm) was employed but they used a specially designed high-temperature column oven which is also based on forced-air convection. However, dual mode retention time predictions using the approach described by Cela and co-workers are less reliable when compared to our approach or the approach presented by Nikitas and co-workers. This could be related to thermal lag phenomena in the HPLC column and the column oven. For example, Nikitas and co-workers introduced a differential equation to take the thermal lag phenomena into account and to improve the accuracy of retention time predictions [13]. Moreover, we could show that thermal lag phenomena can be neglected during retention time predictions using the LES model in temperature-programmed LC if a column oven based on block heating is employed [15]. In contrast, Cela and co-workers used a high-temperature column oven, which is based on the air-bath concept [14]. Furthermore, they do not describe if their approach takes thermal lag phenomena into account. It is possible that this might be the reason for the major errors of dual mode retention time predictions.

Regarding our approach of retention time predictions of simultaneous solvent and temperature gradients it has to be considered that predictions based on this approach strongly depend on the conditions of the measurements which are employed during data fitting. For reliable retention time predictions, the start temperature as well as the percentage of the organic modifier at the start of the temperature and the solvent gradient must be equal to the values of the measurements which are used for data fitting. Otherwise, major errors of predicted retention times will be observed. In other words, to predict simultaneous solvent and temperature gradients where a different start temperature of the temperature gradient is applied and the composition of the mobile phase is not equal to the composition of the mobile phase which is employed during data fitting, at least eight input measurements are required. Moreover, it is difficult to immediately find optimal solvent and temperature gradient conditions that guarantee a successful data fitting using the LES model. This means that the practitioner has to perform several pretest measurements to find out optimal conditions for the eight basic input runs. Hence, some failed attempts will be expected which might increase the quantity of initial basic measurements. In contrast, the dual mode gradient retention model developed by Nikitas and Pappa-Louisi [12,13,30] permits predictions of simultaneous solvent and temperature gradients based on six isocratic/isothermal measurements. They used two temperatures at which measurements were performed at three different organic modifier

percentages. The approach described by Cela and co-workers also uses isocratic/isothermal measurements and in a second stage gradient measurements [14]. Cela et al. recommend three data points in each dimension. In other words, nine isocratic/isothermal runs and at least two solvent and/or temperature gradient input measurements. This means that at least eleven input runs are required. Nikitas et al. and Cela et al. preferred isocratic/isothermal input measurements because predictions based on these data are more precise when compared to predictions on the basis of gradient input measurements due to linear retention assumptions using gradient data. A drawback of the use of isocratic/isothermal input data is that more experimental work is required. For example, if an analyte mixture is considered which consists of polar as well as non-polar analytes, it will be difficult to elute strongly retained compounds at low percentages of the organic modifier in the mobile phase. In other words, an excessive long analysis time or an increase of the quantity of the initial measurements is expected. In contrast, during solvent and/or temperature-gradient elution, the elution strength (polarity) of the mobile phase is continuously increased so that the elution of compounds with different polarity will be possible during the same run.

However, an advantage when using the approaches developed by Nikitas et al. and Cela at al. is that reliable retention time predictions are also possible if the start temperature of the temperature gradient as well as the start percentage of the organic modifier of the solvent gradient is different from the values of the basic input measurements. In addition, problems during data fitting were not reported by Nikitas and Cela.

Summing up, in order to perform as few measurements as possible during systematic method development for simultaneous solvent and temperature programming in LC, the dual mode retention model developed by Nikitas should be employed instead of the approach described during this study. Nevertheless, the approach introduced by Cela and co-workers seems to be useful as well, but prior to further widespread use the reason for the higher relative errors in predicted dual mode retention times needs to be identified and corrected.

Acknowledgment

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Chapter 6. General Conclusion and Outlook

The use of elevated temperature or temperature programming in liquid chromatography (LC) provides several advantages such as fast analysis, increased efficiency, a change of selectivity and an increase of the elution strength of the mobile phase. Method development in high-temperature liquid chromatography is usually performed by trial and error approaches although a systematic approach is required. Therefore, this work was focused on the development of a systematic, computer-assisted approach for method development in temperature-programmed LC. For that reason, the empirical linear elution strength (LES) model was transferred from temperature-programmed gas chromatography to temperature-programmed liquid chromatography. It was shown that the LES model can also be employed to predict retention times of selected analytes depending on a temperature gradient in LC. Moreover, retention time predictions could be calculated very precisely and independent of the column chemistry (silica-based and metal oxide-based columns), inner column diameter (1.0 mm to 4.6 mm) and compound class (steroids, polycyclic aromatic hydrocarbons, sulfonamides, and food additives). Furthermore, the retention times for these classes of compounds were predicted for large temperature intervals ranging from 40 °C up to 180 °C where the temperature gradient slopes were varied between 1.5 °C min^{-1} and 30 °C min^{-1}. For these retention time predictions an average relative error of less than 5% was observed. In addition, a set of recommendations was established to assist the user during systematic method development in liquid chromatography.

Moreover, in this work isothermal retention time predictions based on temperature gradient as well as isothermal input data were investigated. Using the classical approach by means of a linear regression of a plot of $\ln k$ vs $1/T$, curved plots were observed whereas a strict linear behavior was expected. The aim of this work was not to investigate why curved plots of $\ln k$ vs $1/T$ will be observed. However, this might be a topic for further investigations, because a deeper understanding of the reasons for curved plots of $\ln k$ vs $1/T$ would facilitate the choice of a suitable mathematical relationship to predict isothermal retention times.

In addition, further work should focus on the development of a broader variety of temperature stable HPLC columns. Up to now, several high temperature stable columns are commercially

available, but a broader variety of column selectivities (e.g. Phenyl, Amino, C_8, C_4) would assist the application of high temperature liquid chromatography.

Furthermore, for a successful implementation of method development software in high-temperature LC, additional studies should focus on the prediction of the peak width depending on temperature gradients, because the main aim of systematic method development in liquid chromatography is the precise prediction of the chromatographic resolution. This simulation requires an exact prediction of the retention time and peak width of the analytes. Using the LES model, the retention times of the analytes can be predicted very precisely. In future studies, it has to be evaluated how the peak width can be predicted depending on temperature gradients in liquid chromatography. Furthermore, these investigations should consider different high-temperature column oven concepts such as forced-air convection column ovens as well as ovens based on block heating.

Regarding retention time predictions of simultaneous solvent and temperature programming in LC, it seems that the approach developed by Nikitas et al. is more suitable or applicable when compared to the methodology described in this work. In the present work, isothermal as well as simultaneous solvent and temperature-gradient input measurements were required to predict retention times for simultaneous solvent and temperature gradients. On the basis of this data set it is not possible to predict retention times of analytes for temperature gradients under isocratic conditions. In contrast, the retention models developed by Nikitas and co-workers make use of isothermal/isocratic input measurements. On the basis of this data set, the retention models permit the prediction of retention times for temperature gradients in isocratic mode as well as simultaneous solvent and temperature gradients. In other words, the same data set can be employed for both prediction approaches. However, it has to be considered that the retention models by Nikitas and co-workers were evaluated for a small temperature interval ($\Delta T = 60$ °C) using temperature-gradient slopes up to 10 °C min^{-1}. Therefore, the retention models described by Nikitas and co-workers should be evaluated using a larger temperature interval (e.g. 40 °C to 180 °C) and higher temperature-gradient slopes (e.g. 30 °C min^{-1}). Moreover, these investigations should be performed using a high-temperature column oven such as the HT-HPLC 200 by SIM (Scientific Instruments Manufacturer GmbH) or the Polaratherm™ column oven by Selerity Technologies. In addition, the retention models described by Nikitas and co-workers have been extended by a differential equation to take thermal lag phenomena in the HPLC column and in the column oven into account and to improve the accuracy of predicted retention times. This

mathematical extension was necessary because of the employed forced-air convection column oven during their studies. Therefore, it should be investigated whether this extension is necessary if a high-temperature column oven based on block heating is used. It is thinkable that in this case, the retention models yield also reliable retention time predictions without the extension to take thermal lag phenomena into account. In other words, it seems possible that the retention models can be simplified.

Overall, the presented work clearly underlines that the adapted linear elution strength model is well suited to assist the practitioner during systematic method development in temperature programming liquid chromatography. In other words, using the hyphenation techniques where temperature programming is absolutely mandatory such as LC taste or LC-IRMS, the user has now a powerful tool for a systematic and time-saving method development.

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List of Abbreviations and Symbols

ACN	acetonitrile
ACS	American Chemical Society
A-DNPH	aldehyde-2,4-dinitrophenylhydrazones and ketone-2,4-dinitrophenyl-hydrazones
BEH	ethylene bridged hybrid
DAD	diode array detector
ELSD	evaporative light scattering detector
ESI-MS	electrospray-ionization mass spectrometry
et al.	et alii
exp. RT	experimental retention time
GC	gas chromatography
HPLC	high performance liquid chromatography
HPLC-IRMS	high performance liquid chromatography – isotope ratio mass spectrometry
HT-HPLC	high-temperature liquid chromatography
ID	inner column diameter
IRMS	isotope ratio mass spectrometry
LC	liquid chromatography
LC-IRMS	liquid chromatography – isotope ratio mass spectrometry
LES	linear elution strength
LSS	linear solvent strength
MeOH	methanol
MS	mass spectrometry
OD	outer column diameter
p. a.	pro analysi
PAH	polycyclic aromatic hydrocarbon
PBD	polybutadiene
pH	potentia hydrogenii
pred. RT	predicted retention time
psi	pound-force per square inch
R	resolution
RF	refractive index
RMSE	root-mean-square error
RP	reversed phase
RP-LC	reversed phase-liquid chromatography
R_S	critical resolution
SIM	Scientific Instruments Manufacturer

SP	service pack
UV	ultraviolet
v/v	volume to volume

b_ϕ	solvent gradient-steepness parameter
b_T	temperature gradient-steepness parameter
ΔH	enthalpy of transfer of a solute from the mobile into the stationary phase
k_0, k_i	retention factor of a solute corresponding to the start conditions
$k_{0\phi}$	value of the retention factor of a solute at the start of the solvent gradient at constant temperature
k_{0T}	value of the retention factor of a solute at the start of the temperature gradient at constant mobile phase composition
$k_{e,\phi}$	value of the retention factor of a solute at elution during solvent gradient elution at constant temperature
$k_{e,\phi,T}$	value of the retention factor of a solute at elution during simultaneous solvent and temperature gradient elution
r	frictional migration of a solute across the column
R	ideal gas constant
ΔS	entropy of transfer of a solute from the mobile into the stationary phase
S_ϕ	solvent gradient dependent solute constant
S_T	temperature gradient dependent solute constant
ΔT	temperature range ($\Delta T = T_{final} - T_{start}$)
T	absolute temperature, unless otherwise noted
t_0	column dead time
t_D	dwell time of the solvent gradient system
t_{DT}	temperature-dependent delay time
t_G	solvent gradient time
T_G	temperature gradient time
T_{final}	end temperature of the temperature gradient
T_{start}	temperature at the start of the temperature gradient

Greek letters

β	volume phase ratio of the stationary and mobile phase in the column
$\Delta\phi$	solvent gradient range ($\Delta\phi = \phi_{final} - \phi_{start}$)
ϕ_{final}	final concentration of the organic modifier in the mobile phase at the end of the solvent gradient
ϕ_{start}	concentration of the organic modifier in the mobile phase at the start of the solvent gradient

List of Publications

Publications in peer-reviewed Journals

P. Ermisch, T. Teutenberg, S. Wiese, H. Weber, *Determination of suitable column geometries by means of van Deemter- and kinetic plots for method development in high-temperature liquid chromatography isotope ratio mass spectrometry.* Submitted to Analytical Chemistry

S. Wiese, T. Teutenberg, and T.C. Schmidt, *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.* Submitted to Journal of Chromatography A, in revision

T. Teutenberg, S. Wiese, *About the use of acetone as alternative solvent in high-temperature and high-pressure reversed phase liquid chromatography for the separation of derivatisized aldehydes and ketones.* Submitted to Journal of Chromatography A, in revision

S. Wiese, T. Teutenberg, and T.C. Schmidt. *A General Strategy for Performing Temperature Programming in High Performance Liquid Chromatography: Prediction of Segmented Temperature Gradients.* Journal of Chromatography A, 2011, **1218**(39): 6898-6906.

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Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

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Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„A General Strategy for Performing Temperature Programming in High Performance Liquid Chromatography"

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