1 **Temperature-assisted solute focusing with sequential**
2 **trap/release zones in isocratic and gradient capillary liquid chromatography:**
3 **Simulation and experiment**
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Abstract

In this work we characterize the development of a method to enhance temperature-assisted on-column solute focusing (TASF) called *two-stage TASF*. A new instrument was built to implement two-stage TASF consisting of a linear array of three independent, electronically controlled Peltier devices (thermoelectric coolers, TECs). Samples are loaded onto the chromatographic column with the first two TECs, TEC A and TEC B, cold. In the two-stage TASF approach TECs A and B are cooled during injection. TEC A is heated following sample loading. At some time following TEC A's temperature rise, TEC B's temperature is increased from the focusing temperature to a temperature matching that of TEC A. Injection bands are focused twice on-column, first on the initial TEC, e.g. single-stage TASF, then *refocused* on the second, cold TEC. Our goal is to understand the two-stage TASF approach in detail. We have developed a simple yet powerful digital simulation procedure to model the effect of changing temperature in the two focusing zones on retention, band shape and band spreading. The simulation can predict experimental chromatograms resulting from spatial and temporal temperature programs in combination with isocratic and solvent gradient elution. To assess the two-stage TASF method and the accuracy of the simulation well characterized solutes are needed. Thus, retention factors were measured at six temperatures (25-75 °C) at each of twelve mobile phases compositions (0.05-0.60 acetonitrile/water) for homologs of *n*-alkyl hydroxylbenzoate esters and *n*-alkyl *p*-hydroxyphenones. Simulations accurately reflect experimental results in showing that the two-stage approach improves separation quality. For example, two-stage TASF increased sensitivity for a low retention solute by a factor of 2.2 relative to single-stage TASF and 8.8 relative to isothermal conditions using isocratic elution. Gradient elution results for two-stage TASF were more encouraging. Application of two-stage TASF increased peak height for the least retained solute in the test mixture by a factor of 3.2 relative to single-stage TASF and 22.3 compared to isothermal conditions for an injection four-times the column volume. TASF improved resolution and increased peak capacity; for a 12-minute separation peak capacity increased from 75 under isothermal conditions to 146 using single-stage TASF, and 185 for two-stage TASF.

66 **Keywords**

67 Volume overload, on-column focusing, large volume injections, temperature

1. Introduction

To adequately monitor low concentration solutes in small-volume biological samples practitioners turn to gradient elution capillary liquid chromatography (cLC) often coupled to high resolution mass spectrometry. While capillary columns have been effective for proteomics, [1] metabolomics [2-4] and *in vivo* neurochemical measurements [5-10] technological improvements leading to higher concentration solute bands entering the detector are always welcome. Over the last decade major improvements in particle and pump technology have benefitted LC performance [11-15]. The aforementioned improvements in chromatographic performance are important, but do not address the issues related to the often (relatively) large injection volumes used in cLC. The injection of a large enough volume results in so-called volume overload in which early eluting peaks are broadened as a result of inadequate sample focusing at the head of the column [16-20]. Pre-column strategies can be particularly effective because they may involve solute-specific, high-affinity focusing [21-25] or fractionation, e.g., in proteomics (MudPIT [26, 27]). On-column focusing or preconcentration is a consequence of generating transient conditions during the injection that result in high solute retention at the head of the column [28]. For example, focusing can be induced by injecting aqueous samples onto a reversed phase column or application of a solvent gradient [29]. Such on-column focusing may lack the ability to take advantage of solute-specific focusing approaches such as SPE, but it is very convenient, performed on a single column and widely applicable. On-column focusing at the column head focuses all analytes in the mixture in contrast to post-column trapping and remobilization approaches that enhance signal for one or two specific solutes in the mixture [30-33]. One process that would be difficult to accomplish using online solvent-based focusing is to amplify the effect by carrying out the focusing process twice, in series, on the same column.

The small size of cLC columns makes them more susceptible to extra column dispersion and volume overload but their low thermal mass makes the application of dynamic temperature changes chromatographically useful [34]. Temperature programming as well as various temperature pulsing techniques are effective for increasing separation speed and tuning chromatographic selectivity in cLC [35-47]. Cooling cLC columns to sub-ambient temperatures is also advantageous. The Greibrokk group has done extensive work in this area with temperature programs initiated near 5 °C [48-52]. Recently, Schoenmakers et al. immersed a capillary monolith in ice water to induce an in-column solvent switch [33]. In addition to our work with temperature-assisted on-column solute focusing (TASF) [53-55], Holm et al. [56] and Eghbali et al. [57] used segmented column cooling to focus solute bands at specific times and locations along the column, the former the column inlet, the latter the outlet. Dynamic changes in column temperature can be used effectively to manipulate solute bands on column and address the volume overload and dispersion problems.

In our previous work just mentioned we controlled the temperature of a single, one-cm long zone at the head of a capillary column to focus injected solute bands by cooling. Heating the zone lowers the retention factor, sending the compressed injected band down the column. In principle, this process could be repeated to achieve further focusing in a second, adjacent temperature-controlled zone. Here, we describe the development of this idea. We have termed this approach *two-stage TASF*. Our goal is to understand in detail this two-stage TASF approach. Of critical importance, we have developed a straightforward but powerful simulation of the effect of changing the temperatures in the two zones on retention, band shape, and band spreading. The simulation uses a simple stepwise modeling procedure similar to that of Gilar et al. [58, 59]. The Gilar et al. approach is simpler than the more rigorously formulated approach based on mass balance [60, 61] but it does not calculate on-column dispersion. In the mass balance case, on-column dispersion must be handled in an *ad-hoc* fashion because of the Craig-tube-like spreading that occurs naturally in the computation. We have combined the approach of Gilar et al. in which the front and rear positions of each individual zone are calculated, with a straightforward approach to on-column dispersion. Unlike earlier approaches [58, 60-64] we explicitly include the time and space dependence of retention and dispersion. Thus, this simulation is capable of predicting chromatograms resulting from spatial and temporal temperature programs in combination with isocratic and solvent gradient elution.

For simulation of experimental chromatograms, solvent- and temperature-dependent retention data are needed. We determined retention factors at six temperatures in each of twelve mobile phase compositions for a series of *n*-alkyl hydroxybenzoate esters and *n*-alkyl *p*-hydroxyphenones. These model solutes were used to assess the instrument's operation in both isocratic and gradient modes. Our understanding of the processes involved was assessed by comparison of simulated chromatograms with no adjustable parameters to actual chromatograms.

2. Instrumentation and chromatographic conditions

2.1 Chemicals

Uracil, methyl, ethyl, *n*-propyl, and *n*-butyl esters of *p*-hydroxybenzoate (parabens, notated here after as PB1, PB2, PB3, and PB4) were purchased from Sigma-Aldrich (St. Louis, MO). *p*-Hydroxy *n*-alkyl phenones, 1-(4-hydroxy phenyl) ethanone, 1-(4-hydroxy phenyl) propanone, and 1-(4-hydroxy phenyl) butanone (HP2, HP3, and HP4) were from TCI America (Philadelphia, PA). Standard solutions for each paraben and hydroxyphenone were made by dissolving each individually in acetonitrile. Uracil stocks were made in deionized water. Water was from a Millipore Milli-Q Synthesis A10 water purification system (Billerica, MA) and used without further treatment. Acetonitrile (LC/MS Optima grade), isopropanol (HPLC grade), acetone (HPLC grade) and phosphoric acid (HPLC grade) were from Fisher Scientific (Fair Lawn, NJ).

2.2 Instrumentation

2.2.1 Solute retention studies

A Jasco X-LC 3000 system consisting of a 3059AS autosampler, dual 3085PU semi-micro pumps, 3080DG degasser, 3080MX high pressure mixer, CO-2060 thermostated column compartment, 3177UV variable wavelength UV absorbance detector, and LC-Net II/ADC from Jasco Inc. (Easton, MD) was used to evaluate the temperature and solvent composition dependence of solute retention. Data were acquired using ChromNAV version 1.19.3 software. Chromatograms were exported and analyzed using a peak finding program written in MATLAB 2015a (MathWorks, Natick, MA).

2.2.2 Two-stage temperature assisted solute focusing

A diagram for the instrumentation used for two-stage TASF is shown in Fig. 1. Hardware was similar to that used for single-stage TASF [53, 55]. In this instrument we constructed an array of three independently controlled 1.0 x 1.0 cm, 10.9 W thermoelectric elements (Peltiers, TECs) from Custom Thermoelectric (04801-9330-34RB, Bishopville, MD). Each TEC, labeled TEC A, B, C from the column inlet, was silver soldered to a custom liquid cooled copper heat sink mounted inside a polyvinylchloride housing. Heat sink temperature was controlled using a Thermo Haake K10 (Newington, NH) digital temperature controller pumping an ethylene glycol/water mixture at about 1 L/minute. TECs were separated from each other by a distance of 100 μ m. Each TEC's temperature was monitored with a 36-gage Type-T thermocouple (Omega Engineering, Stamford, CT) epoxied to its surface. Care was taken to ensure the tip of each thermocouple rested on the TEC surface with no epoxy at the TEC/thermocouple interface. Temperature measurements were made with a National Instruments 9213 16-channel high speed thermocouple input module (Austin, TX). Each TEC was independently controlled by a Maxim Integrated 1968 electronic TEC driver ($V_{\text{max}} = 5$ V, $I_{\text{max}} = \pm 3$ A, Sunnyvale, CA) interfaced with a NI 9264 32-channel analog voltage out module. A simple feedback loop-based LabVIEW program was written in-house to coordinate temperature changes and maintain desired TEC temperature. [55]

The downstream, isothermal segment of the column was heated using a 28 V, 3" long Kapton resistive heater (KH-103-10-P, Omega) and a Love Model 1500 proportional-integral-derivative (PID) controller (Dwyer Instruments, Michigan City, IN) as described previously [65].

Mobile phase was delivered by a Thermo/Dionex UltiMate 3000 Nano LC (NCS-3200RS, Germering, Germany) capillary pump. The pump was connected to an externally mounted 6-port two-position Cheminert injection valve (C72x-669D, VICI Valco, Houston, TX) by a 55 cm x 50 μ m I.D. nanoViper capillary (Thermo). A second 75 cm x 75 μ m I.D. nanoViper capillary (3.3 μ L volume) was used as the injection loop.

185 Injected volumes were determined by using timed injections with valve actuation
186 controlled by the same LabVIEW routine used for TEC temperature control. Remote
187 start and valve switching signals were coordinated by an NI USB-6008 DAQ. Fused
188 silica capillary columns (packing described below) were connected directly to the
189 injection valve using 0.015" I.D. x 1/16" O.D. PEEK sleeves from IDEX-Health and
190 Science (Oak Harbor, WA). A Waters Acquity TUV fitted with a 10 nL nano flow cell
191 (Waters Corporation, Milford, MA) was used for detection. A 25 cm long 25 μ m I.D. x
192 360 μ m O.D. fused silica detection capillary was placed between the column outlet and
193 flow cell; connections were made using PTFE unions. Data were acquired at 100 Hz
194 using either an Agilent 1200 Series Universal Interface Box (Agilent Technologies,
195 Waldbronn, Germany) and ChemStation OpenLab CDS (C01.06) software or an Atlas
196 A2D and Chromeleon version 6.8 software (Thermo). All data analysis was performed
197 in MATLAB 2015a using peak evaluation and integration programs written in-house.

2.3 Chromatographic conditions

2.3.1 *van't Hoff retention studies*

200 Temperature-dependent retention factors for parabens and *p*-hydroxyphenones were
201 determined on a new Waters Acquity BEH C18 (50 mm x 2.1 mm I.D., 1.7 μ m d_p)
202 column. Samples were made in mobile phase at concentrations between 100 and 250
203 μ M. Column temperature was varied from 25 to 75 °C in 10 °C steps at each mobile
204 phase composition. Compositions tested were: 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35,
205 0.40, 0.45, 0.50, 0.55, and 0.60 (w/w) acetonitrile/10 mM H₃PO₄. Each mobile phase
206 was prepared by weight to improve composition accuracy and minimize
207 inconsistencies in intersystem mobile phase blending. Weight-to-weight aqueous-
208 organic compositions were converted to volume-to-volume units using density data
209 provided by Billen et al. [66]. Detection at 100 Hz used absorbance at 235 and 254 nm;
210 injection volume was 5 μ L. Flow rate was set to 0.35 mL/min. Retention times were
211 adjusted for extra column time determined using a Valco zero-dead-volume union in
212 place of the column. Table SI 1 shows the solutes used at each mobile phase
213 composition and conversion of mobile phase composition from w/w to v/v units.

2.3.2 *Two-stage temperature assisted solute focusing*

2.3.2.1 *Column preparation*

216 Fused silica capillary columns were packed in-house using a previously described
217 procedure [28, 53, 55]. Briefly, Waters Acquity BEH C18, 1.7 μ m d_p , particles were
218 packed into 150 μ m diameter fused silica capillaries from Polymicro Technologies
219 (Phoenix, AZ). Column blanks were fritted using electrically sintered 2 μ m solid
220 borosilicate spheres (Thermo Scientific, Fremont, CA). Particle suspensions were
221 prepared in isopropanol at 65 mg/mL and sonicated for 20 minutes. Columns were
222 packed via the downward slurry method using a Haskel DSHF-302 pneumatic

amplification pump. Acetone was the packing solvent. A maximum packing pressure of 20,000 psi was held for 10 minutes. Following packing, system pressure was allowed to dissipate naturally. After packing the stationary phase bed the remaining length of the capillary was packed with 8 μm solid silica spheres (Thermo) at 21,500 psi for an additional 10 minutes. Columns were flushed with acetonitrile prior to use. The column used for TASF instrument evaluation had dimensions of 78 mm x 0.15 mm I.D. Column fluid volume or dead volume (V_0) was estimated at 750 nL.

2.3.2.2 Two-stage TASF: Isocratic elution

Mobile phase was prepared by mixing 800.00 g of 10 mM H_3PO_4 and 200.00 g of acetonitrile. Samples were made to match the mobile phase composition; an approximately 10 μM sample of PB2, PB3 and uracil was prepared by adding 20 μL of 50 mM paraben stocks to 100.00 g of the premixed mobile phase. The difference between the mobile phase and sample solvent was kept under 0.05%. Flow rate was 3.00 $\mu\text{L}/\text{min}$, with the pump delivering 50:50 channel A/B. Detection wavelength was 254 nm. Injection volume was 1500 nL for all isocratic separations. The injection volume corresponded to about 200% of the column void volume.

The 1500 nL injection resulted in a 30 s injection time; TEC A focusing time was set to 35 s. This value was in line with the focusing times used in our previous work with single-stage TASF [53, 55]. TEC B focusing times were varied systematically from 35 s, corresponding to a single-stage TASF run, to 80 s in 5 s steps up to 70 s. Variable focusing time injections were performed in duplicate. Injections with focusing times for TEC B at 35 and 60 s were performed in triplicate. TEC C was operated isothermally, maintaining a fixed temperature throughout the run. Heat sink temperature was 25 $^\circ\text{C}$. Focusing and separation temperatures for TECs A and B were 5 and 70 $^\circ\text{C}$, respectively. TEC C and the resistive heater were maintained at 70 $^\circ\text{C}$.

2.3.2.3 Two-stage TASF: Gradient elution

Mobile phase for channel A was 10 mM H_3PO_4 in water and channel B was acetonitrile. The linear gradient was initiated at 5% B and increased to 45% B in 16 minutes. Following the gradient, the pump and column were reequilibrated at the initial mobile phase condition for 4 minutes. The flow rate and detection wavelength were 3.00 $\mu\text{L}/\text{min}$ and 254 nm. Samples of 5 μM PB1, PB2, PB3, and PB4, 7.5 μM HP2, HP3, and HP4 and 25 μM uracil were made volumetrically in 5% acetonitrile to match the initial composition of the gradient. Injection volume was 3000 nL corresponding to 400% of the column volume. Focusing times for TEC A and TEC B were 65 s and 65/100 s (single-stage/two-stage TASF). TEC A and B were reequilibrated for 30 s at the focusing temperature prior to the next run. Focusing and separation temperatures were identical to those used in the isocratic work. Injections were made in triplicate under isothermal, single-stage TASF and two-stage TASF conditions.

3. Results and discussion

3.1 Dependence of retention factors on temperature and solvent composition

Retention was measured at twelve mobile phase compositions between 0.05 and 0.60 w/w 10 mM H₃PO₄/acetonitrile and six column temperatures between 25 and 75 °C at each mobile phase composition. Retention factors under each condition were calculated using Eq. S1 with $n = 4$. Solute mixtures were selected for each mobile phase composition to have a practical experimental k' range at 25 °C from 1 to 65.

Retention data were assessed using a two-step procedure. First partial molar enthalpies of retention were determined for each solute at each mobile phase composition using van't Hoff plots (see Fig. S11 and Table S11). Inspection of residuals indicated there was no significant change in retention enthalpy with temperature over the range studied. Linearity allowed extrapolation to the 5 °C focusing temperature and ensured the accuracy of simulation results. Second, we fit a recent equation to predict the influence of changes in solvent composition and column temperature on solute retention [67] to the retention data. This Neue-Kuss equation is shown as Eq. 1:

$$\ln k = -\ln k_{0,T} + \frac{D}{T} + 2 \ln(1 + a\phi) - \left(1 + \frac{D}{T}\right) \frac{B_T \phi}{1 + a\phi}$$

Eq. 1

where T is the temperature in Kelvin, ϕ is the volume-based mobile phase composition, a and D are coefficients to express the influence of solvent and temperature and $k_{0,T}$ and B_T are the coefficients for retention in 100% water and the slope of the retention relationship with solvent composition including temperature effects.

Nonlinear curve fitting was performed using the Solver add-in in Excel. Note that all fitting results used ϕ values that had been converted from w/w to v/v units using the data from Billen et al. [66]. Results from the curve fitting are shown for all solutes in Table. 1. Fits for the parabens and hydroxyphenones were good with R^2 values in excess of 0.9996 for each solute. Fit quality compared favorably with the data presented by Neue and Kuss for the four parabens using a methanol/water mobile phase and the same Waters BEH particle.

3.2 Characterization of two-stage TASF instrument performance

For effective implementation of two-stage TASF, rapid changes from focusing to separation temperature for multiple, closely spaced TECs is critical. Temperature transients must also be precisely controlled to insure reproducibility. To achieve this, commercially available electronic TEC drivers were used to run, high power, low thermal mass TECs with control software written in-house. Each TEC was operated individually using a self-learning feedback program written in LabVIEW and described previously for a single TEC device [55]. The program was designed to deliver a

reproducible temperature profile to the column with maximum and minimum steady state temperatures of 70 and 5 °C.

To demonstrate the effectiveness of the two-stage TASF instrument an example temperature profile for a two-stage TASF run is shown in panel A of Fig. 2 for TEC A (red), B (blue), and C (black). In this example the focusing temperature of 5 °C was held for TEC A for 35 s and TEC B for 60 s. TEC C temperature was maintained at 70 °C throughout the run. What is important to observe from this figure is the response of adjacent TECs to the temperature change of their neighbors. (See Fig. SI4 for plots of the transient portions of Fig. 2.) After 35 s when TEC A changed to 70 °C there is a small increase in temperature for TEC B of about 2 °C. The temperature control feedback loop for TEC B sensed the increase in temperature and pulled TEC B temperature back towards 5 °C. A similar temperature rise in TEC A and C was observed when TEC B temperature was raised, but again the feedback loop compensated for this and the 70 °C temperature was recovered quickly.

The time derivatives of the temperatures of the three TECs are plotted in Fig. 2B. Maximum heating rates for 15 replicate TASF cycles for TEC A and B were 1091 ± 9 and 1242 ± 10 °C/min (5-70 °C). These values compare favorably to the maximum value of 360 °C/min (25-60 °C) previously reported for a similar array-based TEC setup of Collins et al. [68]. Heating rates for our device translated to a time-to-90% of the set temperature of 7.1 and 6.3 s for TECs A and B. Higher maximum heating rates for TEC B were due to the influence of neighboring TECs and the 25 s delay between heating of TEC A and TEC B. During this time heat from the 70 °C TECs A and C diffused to TEC B assisting its rate of increase to the separation temperature. TEC A did not have this assistance during heating, as it had a cold TEC B to one side and room temperature on the other.

One of the advantages to using TECs to control column temperature was their potential for high speed cooling. High speed heating, up to 1800 °C/min (25-125 °C), has been reported using resistive heaters in cLC [38-40], but these devices are limited in two ways by their lack of cooling capabilities. First, passive cooling restricts the absolute cooling rate to about -150 °C/min (125-25 °C) [39] and second, it does not allow for use of temperatures below ambient. Various other cooling methods such as submerging the column in ice water or blowing compressed gas over it have been reported [33, 41], however these methods are not practical for automated systems necessary for routine use. Using our TEC-based platform, maximum cooling rates of -1485 °C/min (70-5 °C) were achieved for TEC B. This value is nearly ten-times as fast as those reported for passive cooling and over four-times faster than that reported by Collins et al. [68] for TECs (60-25 °C). Performance figures of merit for the two TASF TECs and isothermal

TEC C have been organized in Table 2. Data for TEC C has been included to indicate the isothermal performance during operation of the two-stage system.

3.3 Simulating two-stage temperature-assisted focusing

3.3.1 Development of simulation procedure

In our earlier work on TASF [54] we described a simple model to predict separations using single-stage TASF in isocratic elution. Using the same basic idea, here we present a more powerful digital simulation capable of modeling results from instruments with multiple TECs, each subjecting a section of the column to a unique temperature profile during either isocratic or solvent gradient elution. The simulations, 1) validated the effectiveness of two-stage TASF, 2) provided insight into what is actually happening inside the column and 3) can assist in method development for now more complicated experimental setups.

We predict the position(s) and shapes of the leading and trailing edges of a solute band. First, consider their positions on column and the elution profile in time. In two-stage TASF and in solvent gradient elution these edges have different velocities that are a function of location, z , within the column and time, t , into the run. First, we create a time- and distance-dependent column temperature profile, $T(t,z)$. Second, we determine a time- and distance-dependent solvent profile, $\phi(t,z)$, that incorporates the sample solvent composition, gradient dwell time, initial and final gradient compositions. Local retention factors are calculated at each t and z based on the Neue-Kuss equation which uses $T(t,z)$ and $\phi(t,z)$. Eq. 2 shows the version of the Neue-Kuss equation used in the simulation:

$$\ln k_{loc,i} = -\ln k_{0,T,i} + \frac{D_i}{T(t,z)} + 2 \ln(1 + a_i \phi(t,z)) - \left(1 + \frac{D_i}{T(t,z)}\right) \frac{B_{T,i} \phi(t,z)}{1 + a_i \phi(t,z)}$$

Eq. 2

where $k_{loc,i}$ is the local retention factor for solute i , $T(t,z)$ and $\phi(t,z)$ are values for temperature and mobile phase composition at specific times and locations within the column, $k_{0,T,i}$, D_i , a_i , and $B_{T,i}$ are solute dependent numerical parameters resulting from the fit of the Neue-Kuss equation to our data (Table 1).

From the local retention factors the local elution velocity, $u_{i,loc}$, is determined for each edge of the band:

$$u_{i,loc} = \frac{u}{1 + k_{loc,i}}$$

Eq. 3

where u is the average linear velocity.

Defining a constant time step, Δt , we can track the movement of the edges of the hypothetical rectangular band from the product of the local elution velocity in Eq. 3 and Δt to obtain a distance, Δz , the band moved during that time. Simulations used a Δt value of 1/15 s. Summing the individual distance and Δt values provided precise time and location information for each edge of a solute band for a predefined experimental setup. When the leading edge reaches the end of the column, the corresponding elution time is simply the number of steps required to proceed all the way down the column multiplied by Δt . The rectangular band width, $w_{i,l}$, in length units is determined from the on column distance between the leading and trailing edges of the band when the leading edge elutes. To convert the on-column width to a time-based width, $w_{i,l}$ is multiplied by the well-known post column expansion term:

$$w_{i,t}^2 = w_{i,l}^2 \frac{(1 + k_{eff,i})^2}{u^2}$$

Eq. 4

where $k_{eff,i}$ is the solute retention factor at elution and u is the average velocity.

Next, consider the shape of the zone. Peak shape depends on injection volume and dispersion. The front and rear edges of the injected zone correspond mathematically to the edges of a rectangle that is the sum of two Heaviside step functions. The width of this rectangle is determined as explained above. Each edge is a convolution of a Heaviside step function and the column transfer function. We use a Gaussian as the column transfer function with length standard deviation, $\sigma_{l,i}$. During each step the band spreads according to its local value for plate height, H_{loc} :

$$H_{loc,i} = H(z, t) = \frac{\Delta \sigma_{l,i}^2}{\Delta z}$$

Eq. 5

We use $\phi(t, z)$, $T(t, z)$, values from the solvent and temperature programs to calculate each solutes' local diffusion coefficient, $D_{m,loc,i}$, and reduced velocity, $v_{loc,i}$. Local values for diffusion coefficient were calculated by converting previously reported diffusion coefficients, $D_{m,i,T'}$, at temperature, T' , to the local conditions, $\phi(t, z)$, $T(t, z)$, using the following equation:

$$D_{m,loc,i}(\phi(t, z), T(t, z)) = D_{m,i,T'} \frac{T(t, z)\eta(T')}{T'\eta(T(t, z))}$$

Eq. 6

Local values for mobile phase viscosity as a function of temperature and composition were calculated using the correlation of Guillaume et al. [69] fit to viscosity values for acetonitrile water mixtures from Billen et al. [66]:

$$\eta(\phi, T) = 10^{[-2.533 + 742/T - 0.452\phi + (235/T)\phi + 1.573\phi^2 - (691/T)\phi^2]}$$

Eq. 7

Values calculated for $D_{m,loc,i}$ and $v_{loc,i}$ were inserted into the dimensionless reduced van Deemter equation to determine $\Delta\sigma_{loc,i}^2$ for each particular value of Δz :

$$\Delta\sigma_{loc,i}^2 = \Delta z_i d_p \left[A + \frac{B}{v_{loc,i}} + C v_{loc,i} \right]$$

Eq. 8

where d_p is the particle size and A , B , and C are dimensionless reduced van Deemter coefficients measured by Zhang et al. [70].

Values for $\Delta\sigma_{loc,i}^2$ calculated along the way are summed to determine a variance due to column processes, $\sigma_{l,col,i}^2$ when the band elutes from the column. Converting length to time units results in:

$$\sigma_{t,col,i}^2 = \sigma_{l,col,i}^2 \frac{(1 + k_{eff,i})^2}{u^2}$$

Eq. 9

Sternberg solved the convolution integral for a rectangular pulse and Gaussian distribution [71] for the case where there is no focusing. In the presence of focusing, the injection width in time is not the same as the band width at the exit. Incorporation of the focusing effect yields the following equation for the concentration profile of the observed band:

$$C(t) = \frac{C_0 w_{inj,t}}{2w_{i,t}} \left[\operatorname{erf} \frac{w_{i,t} - (t - t_{R,i})}{\sqrt{2}\sigma_{t,col,tail,i}} + \operatorname{erf} \frac{(t - t_{R,i})}{\sqrt{2}\sigma_{t,col,lead,i}} \right]$$

Eq. 10

following substitution of values from Eq. 4; where C_0 is sample concentration, $w_{inj,t}$ is the injection width in time (the same for all solutes, i), $\sigma_{t,col,lead,i}$ and $\sigma_{t,col,tail,i}$ are the Gaussian standard deviations calculated from Eq. 9 for the leading and trailing edges of the band and $t_{R,i}$ is solute retention time. Note that in Eq. 10 erf represents the error function.

3.3.2 Simulating two-stage TASF with isocratic elution

To judge the effectiveness of two-stage TASF and as an initial validation of our simulation procedure we simulated the separation of a mixture of uracil (void marker), PB2 and PB3 under isocratic conditions. The results from this simulation are shown in Fig. 3. Panels A, B and C illustrate the distance traveled at any time for the solute band

under the three temperature conditions: isothermal (black), single-stage TASF (blue) and two-stage TASF (red), respectively. An overlay of the simulated chromatograms is in panel D. Column dimensions were 80 mm x 0.15 mm ID, 1.7 μm d_p . Injection volume was 1500 nL, flow rate was 3 $\mu\text{L}/\text{min}$, mobile phase composition was 0.20 (w/w) water/acetonitrile, with focusing and separation temperatures of 5 and 70 $^{\circ}\text{C}$. Focusing times for TEC A/B in the single stage TASF and TECs A/B in the two-stage TASF simulations were 35, 35/60 s, respectively. All other chromatographic conditions used in the simulation are in Table S2.

From the spatial representations we can clearly see how TASF and particularly two-stage TASF can be used to transiently modulate solute retention in chromatographically beneficial ways. In each spatial representation, sample is injected onto the column from $t = 0$ until $t = 30$ s ($V_{\text{inj}} = 1.5$ μL , $F = 3.0$ $\mu\text{L}/\text{min}$). During the 30 s sample loading time, the leading edge of each solute band traveled down the column at a velocity dictated by its local retention factor. *The width of the injection band on-column is the source of volume overload.* For the simulation the local retention factors for PB2 and PB3 under isothermal conditions were 4.4 and 10.0 at 70 $^{\circ}\text{C}$. The on-column width of the PB2 and PB3 bands following injection under isothermal conditions were 2.82 and 1.38 cm. In the TASF simulation, column temperature for the first 1.0 cm of the column was set to 5 $^{\circ}\text{C}$, so local retention factors for PB2 and PB3 increased to 15.5 and 42.3. The increased local retention factors at the head of the column resulted in a reduction of on-column zone width for the injection plug to 0.92 and 0.35 cm for PB2 and PB3. While volume overload was significantly reduced by the first TASF stage the now narrowed injection zones still were not compressed enough to induce a Gaussian shaped peak profile at the outlet of the column (blue trace in Fig. 3D).

Addition of the second focusing segment in two-stage TASF allowed solute loaded onto segment one (TEC A) to be released and focused again on the second 5 $^{\circ}\text{C}$ column segment (TEC B). This second focusing effect is clearly visible in Fig. 3C from about 0.5 to 1.0 min as the trailing edge of both PB2 and PB3 bands velocity increased with respect to the leading edge traveling in the cold second segment. The second focusing stage reduced solute band width further and increased overall peak height for PB2 and PB3 by factors of 2.6 and 2.1 relative to the single-stage TASF separations. Decreased peak width improves the overall peak capacity and resolution of the separation while increased peak height benefits analysis sensitivity and quantitation figures of merit.

3.3.3 Simulating two-stage TASF using solvent gradient elution

Now we extend the scope of our simulation procedure to the more complicated system of solvent gradient elution. Fig. 4 shows the simulation for the separation of, in order of elution, uracil (void), HP2, PB1, HP3, PB2, HP4, PB3 and PB4 under isothermal, TASF and two-stage TASF conditions. Darker tones in the spatial representations correspond

to the paraben homologs, while the lighter fill colors represent the hydroxyphenones. Column dimensions were identical to the isocratic simulations. Injection volume was 3000 nL, flow rate was 3 μ L/min, focusing and separation temperatures were again 5 and 70 $^{\circ}$ C. The solvent program was a sixteen-minute linear gradient of water/acetonitrile from 5-45% acetonitrile. Samples were made in 5% acetonitrile; dwell time was 15 s. The focusing time for the TASF separation was 65 s, with the two-stage separation using 65 and 100 s focusing times for TEC A and TEC B. All other chromatographic conditions used in the simulation are in Table S3.

Fig. 4A shows the spatial representation for the isothermal separation. Note that even when using solvent gradient elution with its gradient compression effect the large sample volume and relatively low retention factors for the early eluters still generated significant volume overload for the separation. For clarity, retention factors for each solute under the initial gradient conditions, k_1 , and at the time of elution, k_{eff} , are provided in Table 3. Volume overload is clearly visible for HP2, PB1, HP3, PB3 and HP4 bands as each has a significant width at elution.

As the most chromatographically interesting section of the representation is located in the first 2.0 cm of the column and initial few minutes of the simulation just the portion within the red square (4 minutes, 2.5 cm) of Fig. 4A are plotted for the isothermal, TASF and two-stage TASF in panels B, C and D of Fig. 4. Fig. 4C clearly shows how the addition of the 5 $^{\circ}$ C focusing segment benefited the separation by increasing k_1 for all solutes. While still not compressing the HP2 band width enough to create a Gaussian peak, width at the outlet of the column was reduced significantly, now 0.90 cm compared to 3.70 cm under isothermal conditions. Fig. 4D highlights the importance of the second focusing segment where the 100 s TEC B focusing time was long enough to allow the leading edge of the HP2 and PB1 bands to enter this segment, get refocused and be released as a now narrower band. *Both the HP2 and PB1 bands are focused twice.* Addition of TEC B reduced band width at the outlet of the column to 0.22 cm for HP2. Note that two-stage TASF is a somewhat targeted technique as only solute bands moving fast enough to enter the second cold zone prior to TEC B's heating get focused twice. Low velocity bands, like PB3 and PB4 do not encounter the second focusing zone at all. Fortunately, these slow moving bands focus well enough on their own, due to large k_1 values, and do not need two-stage TASF to produce acceptable results. This is described in more detail in the discussion of experiments below.

Fig. 4E shows an overlay for the simulated chromatograms under the three temperature conditions. TASF (blue) and specifically two-stage TASF (red) work to improve peak shape, height and overall separation performance for low retention solutes. Using TASF the initial retention factor for HP2 and PB1 were transiently increased from 7.2 and 18.5 at 70 $^{\circ}$ C to 32.7 and 106 at 5 $^{\circ}$ C. These relatively small

changes and the spatial temperature program introduced using two-stage TASF increased peak height by factors of 19.9 for HP2 and 10.7 for PB1 relative to the isothermal control.

3.4 Two-stage TASF experiments: Isocratic elution

A series of 1500 nL injections of PB2 and PB3 was made under isothermal, TASF, and two-stage TASF conditions. Sample volume corresponded to 200% of the column fluid volume; sample composition was fixed such that its composition matched the elution strength of the mobile phase. Thus, any improvement in peak shape, height, or width were due to only single- or two-stage TASF. Focusing and separation temperatures for TECs *A* and *B* were 5 and 70 °C, respectively. TEC *C* and the resistively heated section of the column temperature were maintained at 70 °C. TEC *A* focusing time was 35 s. TEC *B* focusing times were varied from 35 to 80 s in 5 s steps (up to 70 s).

Panel A of Fig. 5 shows the signals obtained from the isocratic two-stage TASF runs with the nine different TEC *B* focusing times. What is clearly visible from the overlay is peak height increased for both PB2 and PB3 as TEC *B* focusing time was increased up to a maximum (55 s, black trace) when peak height began to decrease for PB2. Panels B and C highlight these transitions by plotting the sections of chromatogram corresponding to PB2 and PB3. The primary objective for two-stage TASF was to maximize concentration sensitivity, thus the optimum experimental conditions yielded maximum detector response. Close examination of the 55 s TEC *B* focusing time (black trace) chromatogram in panels B and C show the optimum focusing time for PB2 did not correspond to the optimum focusing time for PB3. The optimal focusing time for PB2 did not provide enough time for the PB3 band to enter TEC *B* prior to heating. The optimum focusing time for PB3 was 70 s (purple trace), a value unacceptable for an analysis including PB2 as portions of the band began exiting the TEC *B* before its temperature rise negating the benefits of TASF. Differences in optimum TEC *B* focusing time for PB2 and PB3 were due to their difference in retention and elution velocity.

Optimal focusing time for TEC *B* represented a compromise between the peak shape for PB2 and PB3. In this example maximum peak height was desired for *both* solutes so a small sacrifice in absolute sensitivity for each was made. Optimal focusing time for the conditions evaluated was determined to be 60 s. For reference this forcing time is shown in red for each chromatogram in Fig. 5.

Fig. 6 shows example chromatograms for isothermal (black), TASF (blue), and two-stage TASF (red) using the optimal TEC *B* focusing time for the separation of uracil, PB2, and PB3. Chromatographic conditions were identical to those used in the optimization study. Not surprisingly single-stage TASF increased peak height for PB2 and PB3 by factors of 3.4 and 4.3, relative to isothermal conditions. Note that single-

stage TASF also decreased peak width by factors of 3.6 and 4.6. For symmetrical peaks it is expected improvements in peak height be followed by equivalent reductions in peak width. The marginally larger relative improvement in width for TASF peaks was indicative of the potential for TASF to reduce peak tailing and improve peak symmetry. A similar effect was observed in our previous work using single-stage TASF with small volume injections where TASF was shown to significantly reduce tailing relative to isothermal analyses [54]. PB2 peak height was increased by a further factor of 2.3 using two-stage TASF and peak width decreased by an additional factor of 2.4 relative to single-stage TASF separation. Improvements for the PB3 peak using two-stage TASF were similar to those for PB2, peak height increased by a factor of 2.0, peak width decreased by a factor of 2.2. Clearly, when used appropriately a second TASF stage can induce a multiplicative focusing effect compared to single-stage TASF for large volume injections.

3.5 Two-stage TASF experiments: Gradient elution

To complete our initial assessment of two-stage TASF, we applied the approach to the separation of a 3000 nL sample of three *p*-hydroxyphenones and four parabens using a linear solvent gradient. The sixteen-minute gradient, initiated at 5% acetonitrile, had a final elution strength of 45% acetonitrile. This gradient was performed with samples made in 5% acetonitrile. Separations were conducted under isothermal, TASF, and two-stage TASF conditions. Focusing times for TEC A and TEC B under TASF conditions were 65 s. Under two-stage TASF conditions, focusing times were 65 and 100 s, respectively. All other chromatographic conditions were identical to those used in the isocratic experiments.

Fig. 7 shows an overlay for representative baseline subtracted separations performed under each temperature condition. Isothermal separations are in black, TASF in blue, and two-stage TASF in red. Non-baseline subtracted chromatograms are provided in Fig. S5. Panel A shows chromatograms containing all seven retained solutes plus uracil. Uracil was added to make the 1-minute wide injection plug more visible. The isothermal conditions resulted in unsatisfactory chromatography. It was not until the PB3 peak that isothermal conditions yielded peaks similar to those obtained when using either single or two-stage TASF. Most salient was the performance improvement achieved with the addition of the second TASF stage. Two-stage TASF made the early-eluting HP2 peak now the most prominent feature in the chromatogram.

Fig. 7B shows the first few minutes of the chromatogram containing only HP2. Reducing column temperature from 70 to 5 °C increased k_1 for HP2 from 7.2 to 32.7. Quantitatively, TASF improved peak height for HP2 by a factor of 7.1, relative to the isothermal separation. Addition of the second stage increased peak height further, now by a factor of 22.3 compared to the isothermal separation. Two-stage TASF also outperformed single-stage TASF by a factor of 3.1 for HP2 and reduced its peak width

by a factor of 3.5. Note that HP2 peak width ($w_{1/2}$) for the 3000 nL injection, equal to four-times the column fluid volume, was reduced to only 1.52 s using two-stage TASF.

Fig. 7C focuses on the most hydrophobic solute in the test mixture, PB4. What is most significant from this overlay is the lack of noticeable difference between the isothermal, TASF, and two-stage TASF separations. Application of either TASF approach did not degrade separation performance for the highly retained solutes.

To evaluate the potential improvement in overall separation performance for two-stage TASF separations, values for peak capacity for the separations shown in Fig. 7 were calculated under isothermal, TASF, and two-stage TASF conditions. Peak capacity, n_c , was calculated using the formal definition derived by Grushka [72], shown in Eq. 12:

$$n_c = \int_{t_{R,1}}^{t_{R,last}} \frac{1}{w_{1/2,i}} dt$$

Eq. 11

where $t_{R,1}$ is the retention time for the first solute in the chromatogram, HP2 and $t_{R,last}$ is the retention time for the last solute, PB4. For peak width, $w_{1/2,i}$ is the width at half height for each peak in the chromatogram. The advantage to calculating n_c in this fashion is that the calculation does depend on a uniform peak width throughout the chromatogram. We have also adopted the notion of sample peak capacity from Snyder and Dolan to define limits of integration. [73]. Plots of the inverse peak width against retention time are shown in Fig. 8. The isothermal separation is in black, TASF in blue, and the two-stage separation is in red. The retention time values used for each curve were from those obtained in the isothermal analysis. The single- and two-stage TASF approaches shift early eluting peaks to slightly longer retention times making more space for additional solutes early in the chromatogram, thus the use of retention times from the isothermal analysis was the fairest method for comparison. Trapezoidal integration of the area under each curve yielded peak capacity values of: 75, 146, and 185 for isothermal, TASF, and two-stage TASF separations, respectively. Improvements in peak capacity were most apparent early in the chromatogram, with curves converging as solute hydrophobicity increased. What is interesting is that under the experimental conditions this convergence does not occur until PB4 demonstrating that TASF would improve peak shape for solutes up to those with hydrophobicity equivalent to PB4.

3.6 Assessment of simulation procedure: Comparison to experimental results

The basis for the simulations is laid out in section 3.3.1. It will be helpful to explicitly state here the input parameters required to generate simulated chromatograms. The program requires the following input: injection volume, mobile phase and sample ϕ ,

TEC temperatures and their related switching times, column length, diameter, porosities, particle diameter, dimensionless van Deemter A, B, C coefficients and mobile phase flow rate. These are all easily specified or controlled except for the van Deemter parameters which we took from the literature [70]. For solutes, we experimentally determined the four parameters of the Neue-Kuss equation that define solute k' as a function of mobile phase composition and temperature, and we used estimates of diffusion coefficients at 37 °C and the temperature-dependence of the mobile phase viscosity to calculate diffusion coefficients at other temperatures. There are no adjustable parameters.

Table 4 displays the correspondence between experiment and simulation for the solutes used under all conditions: no TASF, single-stage TASF, and two-stage TASF. The simulated and experimental retention times are very consistent. From this, we gain considerable confidence in several important aspects of this work. One is the value of the four-parameter Neue-Kuss equation *including extrapolation to temperatures lower than those used in establishing the parameters in the equation*. A second is the confirmation that the temperature control *in the column* is fairly good. It would be difficult to determine the temperature inside the column directly. Even if it were possible with a device like a thermocouple, the presence of the thermocouple itself would be a major perturbation. Thus, the accuracy of the simulation across all of the experimental situations is an indirect indication of the accuracy of the temperature control. In one sense, this should not be surprising. Based on thermal mass, the power used to change a TEC's temperature virtually all goes into changing the TEC's temperature, not the column's. Finally, the third observation is that the overall simulation of retention over different temperatures and solvent strengths is accurate. This provides evidence of an effective simulation.

Table 4 also shows experimental and simulated peak half-widths. The agreement is reasonable. Note that we used values of A, B, and C (dimensionless) from the literature which were based on the same stationary phase chemistry and particle, but a different column diameter, 2.1 mm ID, and chromatograph. This undoubtedly leads to some of the differences noted. The most striking difference is for the very lowest k' solute, HP2, in the gradient runs. We suspect that there may be a secondary mechanism for solute focusing. Figs. 6 and SI5 show significant “dips” in the baseline of TASF chromatograms which we suspect to be due to the changing acetonitrile concentration in the mobile phase resulting from the change in temperature. This pulse of acetonitrile transiently increases the velocity of the trailing edge, enhancing focusing (band compression), but the pulse disperses as it passes down the column. Thus wider zones are more affected, and lower k' species have wider zones at injection than higher k' species.

3.7 Advantages and limitations

We built a device consisting of three, independently controlled variable temperature segments for use in packed-column cLC. We used this device to improve on our previously developed TASF concept, demonstrating the ability of the new design to focus on-column bands twice using the two-stage TASF approach. The three-TEC system also provides more experimental flexibility than our previous systems composed of a single one-cm long focusing segment. The three TEC system can be operated in the single-stage TASF mode with variable length focusing dimensions, namely one-, two- or three-cm long segments. Longer focusing segments facilitate the use of larger injection volumes improving analysis sensitivity without observing band “leakage” from cold to hot column zones. Leakage degrades TASF performance (see Fig. 5). Further, the linear array-based TEC configuration allows for dynamic temperature changes in non-TASF applications. For example, TECs can control retention of selected solutes by controlling temperature at *particular locations* along the column at *particular times*. We view the work presented here using two-stage TASF as a first step towards developing the ability to module retention in a *reliable and predictable* way down the entire length of the cLC column.

While the device presented is a significant advance on our previous single TEC devices, it is not without limitations. The current configuration has discrete 1-cm long focusing zones. Systems composed of smaller, ca. 0.5 cm, variable temperature segments offer certain experimental advantages. The primary advantage to smaller TECs is the ability to use *only the required focusing zone length* for the desired solute, injection volume and focusing temperature. Cooling portions of the column not used for actual focusing wastes system pressure making it unavailable to the practitioner to increase column length or analysis speed. (Pressure is directly related to viscosity and viscosity is temperature dependent, usually increasing with reduction in temperature.) A second limitation of the current design concerns TEC lifetime. TECs in our hands last for hundreds of TASF cycles, but they do wear out eventually becoming unable to change temperature at all. In the current configuration all three TECs are soldered to a single copper heat sink. Repair necessitates the entire heat sink assembly must be disassembled to replace a single malfunctioning TEC. This is the primary limitation of the current design when routine operation is desired. We are in the process of optimizing heat sink design to solve this problem.

4. Conclusions

In this work we have demonstrated through accurate simulation and experiment the potential to focus injection bands *twice on-column* using a process called two-stage TASF. This sequential temperature-based focusing approach presented is not achievable using the more common solvent-based approaches. We have validated its efficacy using simulation and shown experimentally its ability to reduce sample-induced

dispersion in isocratic and gradient elution liquid chromatography. We can draw the following conclusions:

1. Linear arrays of closely spaced TECs can be used effectively to modulate solute retention in a space and time dependent way.
2. The simple simulation procedure based on local retention factors accurately predicts experimental band location and shape under isocratic and solvent gradient elution conditions. Simulations match experiments.
3. The addition of a second TASF stage induces a multiplicative focusing effect when used under isocratic conditions. The effect roughly doubles that observed for low retention solutes under single-stage TASF conditions.
4. Solvent gradient elution also sees significant benefit from the two-stage TASF approach, increasing peak capacity for a simple test mixture by a factor of 2.5 relative to an isothermal control.

These results demonstrate the potential for the broader application of dynamic temperature changes in capillary liquid chromatography to modulate solute retention in chromatographically useful ways using simple, reliable and relatively inexpensive hardware.

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Figure Captions

Fig. 1 Schematic for the column temperature control used for two-stage TASF. Three electronically controlled, one-cm long Peltier elements (TEC A, B, C) were silver soldered to a custom copper liquid cooled heat sink. The remaining segment of the column was heated using a PID-controlled resistive heater.

Fig. 2 A) Typical temperature profiles for TECs A (red), B (blue), and C (black) for two-stage TASF. Focusing temperature was 5 °C, separation temperature was 70 °C; focusing time for TEC A was 35 s and 60 s for TEC B. Small, ca. 2 °C, temperature transients were observed for each TEC due to the temperature change of the adjacent TEC. B) Plot of time derivative of temperature for TECs A, B, and C. Maximum heating rates were greater than 1000 °C/min (for 5-70 °C) for TECs A and B; maximum cooling rates were nearly 1500 °C/min (for 70-5 °C) for TEC B for the specified temperature range.

Fig. 3 A) Spatial representation of isothermal chromatogram for 1500 nL injection of mixture of void marker, ethyl and propylparaben made in mobile phase. B) Same separation under TASF conditions, $T_1 = 5$ °C for 35 s. C) Two-stage TASF separation

demonstrating additional band compression with second focusing stage. D) Simulated chromatogram for isothermal (black), single-stage (blue) and two-stage TASF (red) separations.

Fig. 4 Simulations for gradient elution separations of parabens and hydroxyphenones. A) Spatial representation for complete isothermal gradient elution chromatogram. The void maker is bounded by dashed black lines, hydroxyphenones show as light gray bands, parabens as dark gray bands. B) Region of isothermal spatial representation indicated by red box in panel A highlighting segments of column subjected to temperature changes. C) Single-stage TASF spatial representation for the first 4 minutes of the separation. Focusing time was 65 s. Light blue bands correspond to the alkylphenones, dark blue the parabens. Band width was reduced by additional temperature induced focusing at the head of the column. D) Two-stage TASF, alkylphenones are shown as light red bands, parabens in dark red. *p*-hydroxyacetophenone and methylparaben peaks were clearly focused twice using two-stage TASF. E) Simulated chromatograms for isothermal (black), TASF (blue), and two-stage TASF with 100 s $t_{focus,B}$ time (red).

Fig. 5 A) Example chromatograms from isocratic two-stage TASF study on the optimal TEC B focusing time. TEC A focusing time was fixed at 35 s, TEC B focusing time was systematically increased from 35 s (corresponding to single-stage TASF) to 80 s in 5 s increments. B) Peak profiles for PB2. C) Peak profiles for PB3. See Section 2.3.2.2 for chromatographic conditions.

Fig. 6 Overlay of isocratic chromatograms resulting from 1500 nL injections of uracil, PB2, and PB3 samples under optimal two-stage conditions. Sample composition was made to match the mobile phase composition. Isothermal (black) separations were performed at 70 °C. Single-stage TASF (blue) separations had a focusing time for TEC A and TEC B = 35 s. Two-stage TASF (red) utilized a focusing time for TEC A of 35 s, focusing time for TEC B was 60 s. Focusing and separation temperatures for both TASF modes were 5 and 70 °C.

Fig. 7 A) Overlay of isothermal (black), TASF (blue), and two-stage TASF (red) separations of hydroxyphenones and parabens. B) Excerpt of chromatograms focusing on HP2. Note the significant differences between the isothermal, TASF and two-stage TASF chromatograms. C) Section of the chromatogram containing PB4. Minimal differences between the separation modes is observed indicating the neither TASF approach degraded separation performance.

Fig. 8 Peak capacity for isothermal (black), TASF (blue), and two-stage TASF (red) separations from the example chromatograms shown in Fig. 7.

Figure 1

Figure 2

Figure 5

Figure 6

Figure 7

Figure 8

Table 1. Curve fitting results for Neue-Kuss retention equation using the Waters Acquity BEH C18 column as a function of temperature (25-75 °C) and solvent composition.

Analyte	ln $k_{0,T}$	B_T	a	D	Fit (R^2)	n ^a
p-Hydroxyacetophenone	4.60	2.95	2.42	2559	0.9996	144
p-Hydroxypropiophenone	4.26	2.67	2.03	2878	0.9998	192
p-Hydroxybutyrophenone	4.07	2.65	1.95	3260	0.9998	216
Methylparaben	4.62	2.50	1.96	2890	0.9998	192
Ethylparaben	4.30	2.64	1.99	3271	0.9998	216
Propylparaben	4.24	2.70	2.03	3774	0.9997	216
Butylparaben	4.30	2.73	2.07	4323	0.9996	216

^aTotal number of experimental data points used in nonlinear curve fitting.

Table 2. Two-stage TASF instrumental figures of merit for TEC control, maximum TEC heating and cooling rates, and maximum and minimum overshoot values following the temperature change from 5-70 °C.

Figure of Merit	TEC A	TEC B	TEC C
dT/dt _{Heat,max} (°C/min.) ^a	1091 ± 9	1242 ± 10	-
dT/dt _{Cool,max} (°C/min.) ^b	-1287 ± 13	-1485 ± 8	-
T _{Heat,max} (°C) ^c	71.8	71.1	71.2
T _{Cool,min} (°C) ^d	2.1	2.9	68.6

^{a,b}Mean from 15 replicates, ± SEM.

^{c,d}Maximum and minimum temperatures from 15 separate temperature profiles.

Table 3. Predicted gradient elution retention factors for each solute under isothermal and two-stage TASF conditions.

Solute	$k_1, \phi = 0.05$		$k_{eff}, 70\text{ }^{\circ}\text{C}$	
	70 °C	5 °C	Isothermal	Two-Stage TASF
<i>p</i> -Hydroxyacetophenone	7.2	32.7	5.3	4.0
<i>p</i> -Hydroxypropiophenone	24.1	135	8.0	7.3
<i>p</i> -Hydroxybutyrophenone	77.4	545	9.4	9.0
Methylparaben	18.5	106	7.7	6.7
Ethylparaben	63.9	454	9.1	8.7
Propylparaben	240	2290	9.9	9.7
Butylparaben	910	12000	10.3	10.3

Table 4. Comparison of simulated and experimental retention time and peak width values for test solutes under isothermal, TASF and two-stage TASF conditions using isocratic and solvent gradient elution.

Solute	$t_{R,exp}$ (min.)	$t_{R,sim}$ (min.)	$w_{1/2,exp}$ (s)	$w_{1/2,sim}$ (s)
Isocratic Elution				
Isothermal				
PB2	1.49	1.45	28.8	30.0
PB3	2.82	2.93	28.7	30.0
TASF				
PB2	1.94	1.81	8.1	9.7
PB3	3.22	3.33	6.3	7.6
Two-stage TASF				
PB2	2.16	2.1	3.4	3.6
PB3	3.38	3.5	2.9	2.9
Solvent Gradient Elution				
Isothermal				
HP2	2.25	2.12	39.7	42.0
PB1	3.85	3.83	24.7	25.2
HP3	4.35	4.36	20.5	21.5
PB2	6.38	6.36	10.1	9.34
HP3	6.84	6.80	8.3	7.80
PB3	8.97	8.99	4.5	3.18
PB4	11.43	11.46	3.8	2.72
TASF				
HP2	2.74	2.70	5.4	9.6
PB1	4.13	4.14	4.3	4.4
HP3	4.58	4.58	3.8	3.7
PB2	6.37	6.44	3.4	2.5
HP3	6.83	6.86	3.4	2.5

PB3	8.95	9.02	3.6	2.6
PB4	11.43	11.47	3.8	2.7
Two-stage TASF				
HP2	3.09	2.94	1.5	2.1
PB1	4.25	4.16	2.4	2.0
HP3	4.66	4.58	3.1	3.7
PB2	6.42	6.44	3.4	2.5
HP3	6.88	6.86	3.5	2.5
PB3	8.97	9.02	3.6	2.6
PB4	11.44	11.47	3.8	2.7