Monitoring effective column temperature by using shape selectivity and hydrophobicity and the effects of mobile phase temperature

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Abstract

Relative changes in the hydrophobicity and shape selectivity of standard analytes are shown to be a valuable method to monitor the reproducibility of the effective temperature experienced by the analytes in a chromatographic column in different ovens. Significant differences were observed between ovens with the same nominal temperature and could be a major cause of problems encountered in transferring methods between instruments or laboratories. By using two parameters, changes due to the column temperature can be distinguished from those caused by mobile phase composition differences. In addition marked increases in column efficiency were noted as the mobile phase temperature was reduced below the column temperature in contrast to claims that thermal equilibration is essential.
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1. Introduction

A problem encountered in the transfer of methods between instruments and laboratories has been that, even though the operators may follow the same formal method, the retention and selectivities may differ even on columns from the same batch of packing material. Differences in the temperature of the column are often suspected as a cause and this was particularly evident in interlaboratory studies, when ambient temperatures were used [1]. However, it can be difficult to identify the causes of variations in method transfer because changes in selectivity can be caused by changes in either or both the temperature and mobile phase composition. When critical pairs of analytes are involved even small changes in selectivity can alter resolution and may require method re-optimisation. The examination of the influence of small temperature changes should therefore form part of the robustness testing of any method.

Although in method transfers temperature differences can cause problems, in recent years there has been a greater appreciation of the influence of temperature on selectivity [2,3] and its use in method development and optimisation has been recently reviewed by Dolan [4].

Typically, the hydrophobicity (or methylene selectivity) decreased systematically with increased temperature [5,6]. Other changes can be more complex and the selectivity of pairs of analytes can increase or decrease depending on their structures. For example the NIST SRM 869 [7] shape selectivity test based on benzo[a]pyrene (BaP), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) and phenanthro[3,4-c]-phenanthrene (PhPh) introduced by Sander and Wise [8,9] has been shown to have a significant temperature dependence [10,11]. An alternative shape selectivity test has also been developed by Kimata et al. [12] based on the use of triphenylene and *o*-terphenyl.

In general, increasing column temperature has also been reported to improve column efficiency [13] but it has often been claimed that poor eluent preheating or thermal equilibration of the mobile phase caused band broadening and peak distortion. For example, Wolcott et al. [14] found that reducing the mobile phase temperature compared to the column caused a change in selectivity and the peak shapes of later eluting peaks were distorted and broadened with increased retention. They concluded that the incoming eluent should be within 6 °C of the column temperature when elevated temperatures were used and that there were differences the heat flow in different oven designs. Similar recommendations for thermal equilibration of the mobile phase and column temperatures have been made by other authors, including Carr and co-workers [15]. However, in

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contrast Poppe and Kraak [16] and more recently Mayr and Welsch [17] for analytical columns and Brandt et al. [18] for preparative columns reported that separation efficiency can be increased by cooling the mobile phase to a fixed temperature below that of the column resulting in an inversion of the flow profile. One problem in all these studies is that the column temperature is not uniform because of the heat generated by the resistance to the mobile phase flow and differences in the ability of the medium in the oven (air or water) to conduct the heat away from the column. For example Djordjevic et al. [19] also noted that with an air oven the eluent temperature increased markedly along the column.

The present study set out to examine the effect of small changes in temperature on the hydrophobicity and shape selectivity of a test mixture and then to use the changes to compare the effective column temperature of different ovens and operating conditions. Understanding the influence of these parameters is an essential first step in selecting a protocol for the accurate and reproducible transfer of methods between different laboratories. The work arose as the result of inconsistencies in selectivity and column efficiency observed in a round robin study, which used columns from a single batch of stationary phases and what was thought to be a closely defined method [20] and has subsequently been used to define the protocol for a reference column material for HPLC [21].

2. Experimental

2.1. Chemicals

HPLC grade methanol and acetonitrile were obtained from Fisher Chemicals (Loughborough, UK), uracil was from Sigma (Deisenhofen, Germany) and all other solutes were from Aldrich (Dorset, UK). Water was purified using a Millipore system to $18.2\,M\Omega$.

The mobile phase was 75:25 methanol–water (w/w), and was degassed in an ultrasonic bath before use. The test solution contained uracil 0.020 g/l; butylbenzene 1.0 g/l; pentylbenzene 1.5 g/l; o-terphenyl 0.020 g/l; triphenylene 0.0030 g/l in 2 ml methanol–water 75:25 (v/v).

2.2. Column

The separations were carried out on a custom-made ODS-bonded silica [22] column (5.0 μm particles, 150 mm × 4 mm i.d. packed by Bischoff, Leonberg, Germany.

2.3. Instrumentation

The separations were carried out using a Hewlett Packard HP 1100 system (Waldbronn, Germany) consisting of a binary pump set at a flow rate of 1 ml/min, autosampler, diode array detector monitored at 254 nm and Chemstation. Initial measurements were carried out using the 1100 column oven

but the integral heat exchanger was by-passed. All connections were made with 0.1 mm i.d. PEEK tubing or stainless steel tubing, 0.005 in. i.d. (0.02 mm), 1/16 in. o.d. (Supelco, Bellefonte, USA).

In the later studies the column and/or connecting tubing was placed in a water-bath controlled by a Techne Tempette Junior TE-8J (Cambridge, UK). The oven and column temperatures were measured using a thermocouple and portable temperature meter HI 93530, (Hanna, Bedfordshire, UK), which was calibrated against a certified mercury thermometer (certificate number 6983).

2.4. Calculations

The retention times of the peaks were measured on triplicate or more injections and the shape selectivity and hydrophobicity were calculated as below:

Shape selectivity

$$= \frac{\text{Retention time of triphenylene} - \text{retention time of uracil}}{\text{Retention time of } o\text{-terphenyl} - \text{retention time of uracil}}$$

Hydrophobicity

$$= \frac{\text{Retention time of pentylbenzene} - \text{retention time of uracil}}{\text{Retention time of butylbenzene} - \text{retention time of uracil}}$$

The column efficiencies were calculated by the Chemstation software based on the peak width at half height.

3. Results and discussion

The study was carried out on a custom-made un-endcapped C18 column, which had been prepared by Unger and co-workers [22] as part of a study to develop a certified reference material for HPLC. A test mixture was used, which contained uracil as a void volume marker (Fig. 1). Two alkylbenzenes, butyl- and pentylbenzene, were included as their ratio could be used as a guide to hydrophobicity and the pentylbenzene peak was also used to monitor column
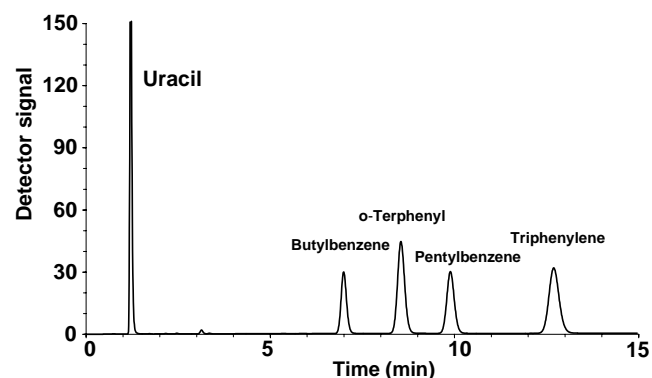

Fig. 1. Separation of test mixture on a ODS-silica column. Conditions: mobile phase methanol–water 75:25 (w:w); flow rate 1 ml/min; temperature 40 °C (water bath); detection 254 nm.

efficiency. The ratio of the retention factors of *o*-terphenyl and triphenylene were used to determine the shape selectivity. To improve the method reproducibility the mobile phase of methanol–water 75:25 was prepared by weight.

3.1. Effect of oven temperature

Initially the column was placed in a standard modular HPLC column oven, which consisted of a thermostatically controlled metal block on which the column rested inside a closed compartment, equilibration of the column being obtained by radiation and convention, but there was no forced air circulation within the oven. The built-in preheating tubing was by-passed and a controlled length of PEEK inlet tubing was placed within the oven as this could be directly compared with other oven configurations. This "metal block" oven was set at 40 °C and a surface mounted thermocouple on the metal block confirmed this value. However, if an empty column was placed in the oven, the internal temperature determined by a thermocouple was only 35.1 °C, suggesting poor heat distribution within the oven. Similar differences were also observed by Zhu et al. [2] and earlier by Paesen and Hoogmartens [23]. They noted that inadequate oven design could generate temperature gradients within an oven and alter the heat transfer rate into and out of the column. In the present study the temperature within the empty column was normally 5–6 °C cooler than the set value of the oven block over the nominal range of 38–50 °C. Significant differences of ±2 °C were also noticed on a day-to-day basis for the same set oven temperature. The measured internal column temperature appeared to be related to the ambient temperature, which ranged from 17 to 25 °C.

During this period five replicate separations of the test mixture were made at a sequence of oven temperature settings from 38.5 to 50.5 °C. As the temperature increased, as expected, the retention times decreased (for example, triphenylene changed from 14.5 min at 38.5 °C to 11.6 min at a setting of 50 °C). However, the mean hydrophobicity and shape selectivity values were poorly reproducible and although there were differences in the slopes of the two parameters with temperature, these could not be determined accurately (Fig. 2). As the temperature setting of the oven increased the column efficiency also increased (pentylbenzene from $N = 72{,}000$ at 38.5 °C to about $N = 83{,}000$ at 50.5 °C) but again there was a considerable variation. It appeared that a significant cause of the uncertainty in the measured parameters might be variations in the effective internal column temperature due to variations in the ambient conditions.

Alternative methods of temperature control were then examined and because this work was part of a study into interlaboratory transferability, methods that could be independent of a particular make of instrument were of particular interest. Static block heaters, without air circulation, were considered a problem because the internal temperature can depend on the extent of contact between the column and oven material.
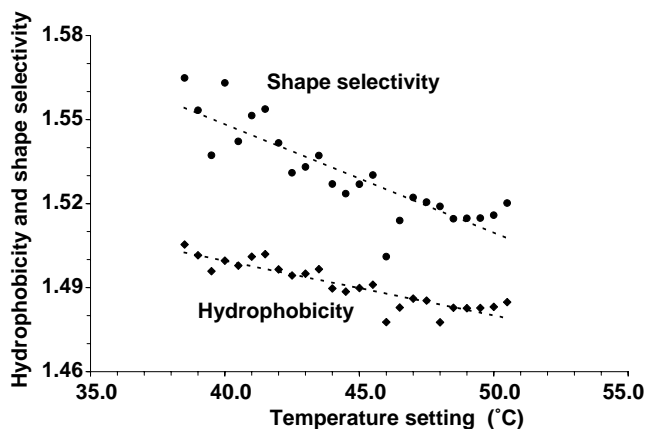

Fig. 2. Variation of shape selectivity (●) and hydrophobicity (■) with temperature setting in "metal block" oven. Inlet tubing PEEK 32 cm.

Hot spots can be generated at the contact points. Circulating air ovens were thought to provide better equilibration but in an interlaboratory study it would be difficult to define an air circulation rate and hence the heat transfer into and out of the columns, without specifying a particular model of oven. Poppe and Kraak [16] also considered that air thermostating was insufficient to maintain a constant wall temperature along a column. Further studies therefore concentrated on stirred water baths, as they have a high thermal mass and good thermal contact between the medium and the column. This should ensure a consistent heat transfer and a uniform external column temperature along its length. The water bath was equipped with a stirrer-heater with a ±0.1 °C response range. A circulating water jacket drawn from a thermostated water bath would have the same effect. In both cases the high thermal mass and good conductivity should ensure that there should be a constant external column temperature and constant heat transfer away from the column irrespective of the ambient conditions.

The column and a defined length of the PEEK inlet tubing were placed in the water bath and the effect of temperature was determined again over the range 35–45 °C. The reproducibility was much higher than earlier. These values were then used to calculate the hydrophobicity and shape selectivity (Fig. 3). Both factors decreased systematically with increasing temperature and the correlation coefficients 0.9935 and 0.9936, respectively suggesting a more stable system. In this system, the efficiencies (pentylbenzene from $N = 56{,}000$ to 64,000) were largely unchanged on increasing the temperature but were significantly lower than in the contact oven even though the tubing and connections were identical.

As the hydrophobicity, and shape selectivity decreased to a different extent with increasing temperature (slopes of hydrophobicity and shape selectivity −0.0033 and −0.0079, respectively), their relative values could be employed as a guide to the effective column temperature of the column (Fig. 4). However, if there was a change in eluent composition, the two values altered in opposite directions.
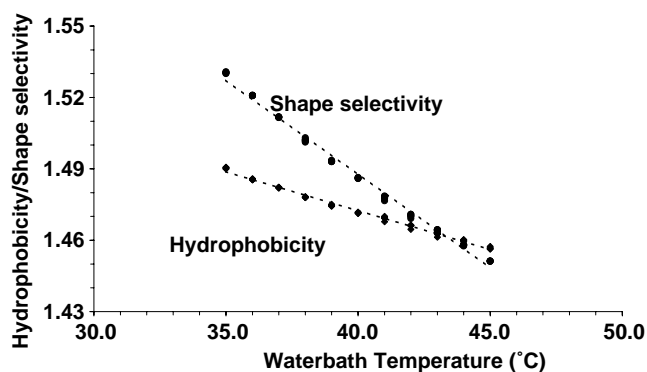
Fig. 3. Variation of shape selectivity (●) and hydrophobicity (■) with water bath temperature (conditions as Fig. 1).

With decreased organic modifier, the hydrophobicity increased but the shape selectivity decreased, for example the values of the hydrophobicity = 1.549 and the shape selectivity = 1.437 in methanol–water 70:30 (w/w) at 40 °C were markedly different from the values of 1.471 and 1.488, respectively, for methanol–water 75:25 (w/w) (Fig. 4).

Thus, if both parameters were determined using identical columns on different instruments, it would be possible to distinguish between the effects of minor changes in temperature and mobile phase composition as the cause of a change in retention time or selectivity. Although these changes have been noted separately previously the two parameters have not previously been compared for the same column.

When different oven types were compared, it was found that the shape selectivity of 1.521 in a water bath at 36 °C was the same as that in a metal contact oven temperature using a nominal setting of 47.5 °C (which gave a internal

temperature for an empty column of 40.9 °C) (Fig. 4). The value of 1.486 for the water bath at 40 °C was lower than the value of 1.52 at the highest setting of 50 °C examined in the contact oven. These differences partly explained why in a round robin trial in which different ovens were used, there had been a wide variation in measured values even though all were set to the same nominal temperature [20]. It appears that the effective column temperature is governed not only by the set column temperature but also by the ability of the oven/surrounding medium to conduct away the energy generated by viscosity induced internal heating.

3.2. Effect of mobile phase temperature

As noted earlier, previous authors have suggested that an equilibrated mobile phase inlet temperature is essential for high efficiency. Using a water bath at 40 °C and a constant length of connecting PEEK tubing of 40 cm, it was found that as the length immersed in the water bath was increased, the temperature of the mobile phase at the exit of the tubing changed from 35.5 °C with 6 cm tubing in the bath to 37.4 °C with 18 cm length. However, when the tubing was connected to the column, as the length in the water bath increased (and hence the mobile phase temperature rose to nearer that of the column) the efficiency of the pentylbenzene peak decreased from 78,000 to 60,000 plates per metre. This change was contrary to reports that the inlet temperature must be equilibrated for maximum efficiency but agreed with Mayr and Welsch [17] and Brandt et al. [18] that a reduced mobile phase temperature produces an inversion of the flow profile and increased efficiency.

To examine the effect of the mobile phase temperature in more detail a second column from the same batch was examined. The PEEK connecting tubing was replaced by a 70 cm length of stainless steel tubing, which would give a more efficient thermal equilibration [17]. A fixed length of 10 cm of the tubing just before the column was placed in a separate water jacket that was independently thermostated. As the mobile phase temperature was reduced from 40 to
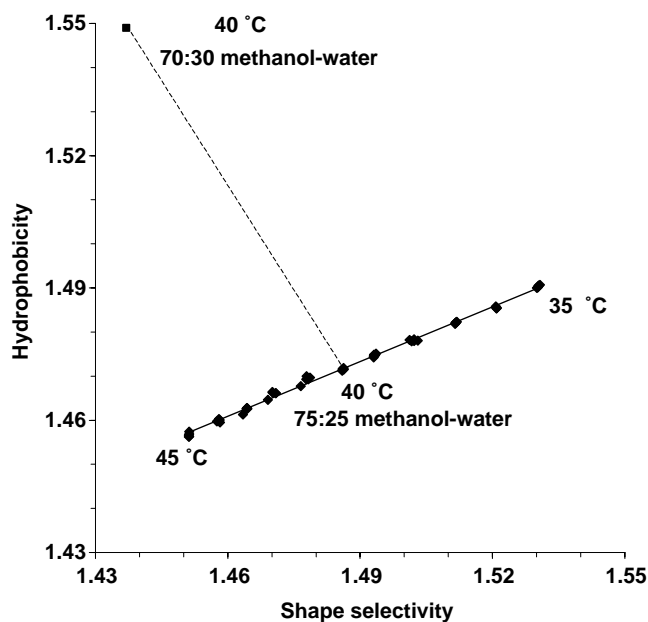

Fig. 4. Comparison of shape selectivity and hydrophobicity at different temperatures with methanol–water 75:25 (w/w) and at 40 °C with methanol–water 70:30 (w/w).
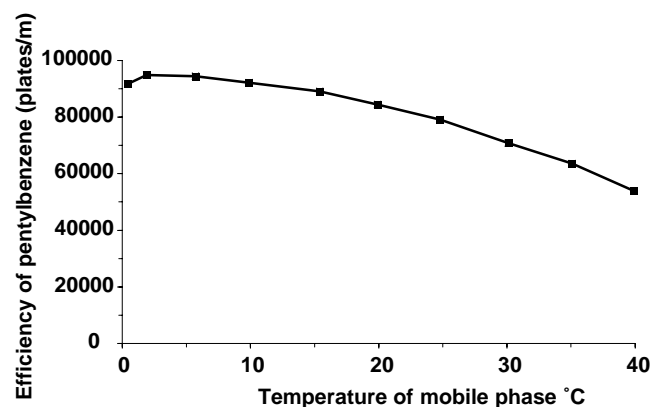

Fig. 5. Effect of temperature of mobile phase from 40 to 0.4 °C on the efficiency of a pentylbenzene peak on a column at 40 °C (other conditions as Fig. 1).
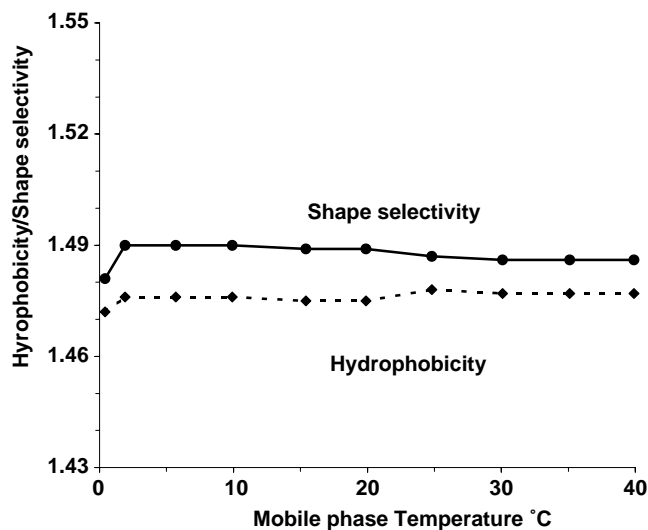
Fig. 6. Effect of changes in the mobile phase temperature, shown in Fig. 5, on the shape selectivity and hydrophobicity of the separation.

$0.4\,°C$, while maintaining the column at $40\,°C$, there was a steady increase in the column efficiency from $N = 53,971$ to 91,722 (Fig. 5). Although a reduction of $10\,°C$ gave the optimum efficiency previously [17], in the present study the improvement continued down to $35\,°C$ below the temperature of the column ($5\,°C$ water bath). Again the shape selectivity and hydrophobicity remained constant (Fig. 6) confirming that the effective overall column temperature was unaltered. Thus any effect from the mobile phase was probably restricted to the first few millimetres of the column.

4. Conclusions

These studies demonstrated that to obtain reproducible separation selectivities that can be reproduced or transferred between laboratories, it is essential to carefully specify the type of oven otherwise the effective column temperature may be different. The variations in the shape selectivities and hydrophobicities of a set of test compounds can then be used as probes of the column temperature and could be specified in a protocol to ensure that the column ovens and conditions being used by different laboratories are generating effectively the same temperature and thermal transfer for the column. As noted by earlier authors, water jackets and contact ovens can yield different results for both efficiency and retention selectivity. The latter indicating a different effective temperature within the column so that for method transfer the type of ovens used could be a critical parameter.

This study also demonstrated that for these columns that an equilibrated inlet temperature will not always give the highest efficiency but that the optimum efficiency was obtained with a cooled mobile phase. This raises the possibility that some reported claims of improved efficiency with increased column temperature might be partly as a result of incomplete mobile phase temperature equilibration causing a temperature differential. This effect is being studied further on a range of column to try to determine why the optimum changes in different studies.

Acknowledgements

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