Turning on the Heat in Liquid Chromatography

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The use of temperature within liquid chromatography (LC) has traditionally been limited to isothermal studies up to 50° or 60°C. However, this does not realise the full potential that temperature can have within a liquid chromatographic system. In particular the ability to run with thermal gradients [1,2] or to run green LC [3,4], where there is no organic solvent used, is something that is not considered as routine within the liquid chromatography community.

The advent of ultra-high pressure liquid chromatography (UHPLC) has meant that chromatographers are becoming more aware of the extremes of chromatography, and the benefits that this can have in terms of the separations either due to efficiency gains or reduction in sample analysis times. The advent of the new phases has also led coincidentally to robustness in the column performance at elevated temperature allowing for more extreme chromatography to be tried. The advantages of this form of extreme chromatography are many-fold e.g. a reduction in viscosity leads naturally to a reduction in the operating pressure, allowing for much higher flow rates and thus reducing analysis times. It also leads to substantial changes in selectivity which can benefit the chromatographer. Interestingly the use of Hypercarb™, which is an ideally suited stationary phase for these extreme conditions, is seeing an increase in the number of applications [5,6].

Experimental

Two different experimental arrangements were employed to demonstrate the benefits of high temperature liquid chromatography. The first set of experiments used a UHPLC column (C18 100 x 2.1 mm, sub 2µm). The chromatographic system comprised of an autosampler and a UHPLC binary pump which was used in conjunction with a GC oven, employed to heat the column, with pre-column heating and post column cooling to ensure optimal performance. This oven was capable of operating at temperatures up to 450°C. The eluent from the column was cooled before it went into a mass spectrometer.

Calibration of the physical parameters associated with the instrumentation has been discussed previously [2], but is essential in determining what experimental flow rates can be used without damage to the instrumentation. It also allows for isobaric studies, where temperature gradients are applied to an isocratic mobile phase to elute components from a HPLC column, but maintaining a constant pressure which is used as a coarse marker to determine the optimum flow for a set temperature.

For the production of the experimental data on Hypercarb there is a change to the experimental arrangement. Instead of a GC oven the Selerity Polaratherm™ Series 9000 oven was employed. This has an inbuilt pre-column heater and also a post column cooler. Instead of a mass spectrometer, a UV detector is used.

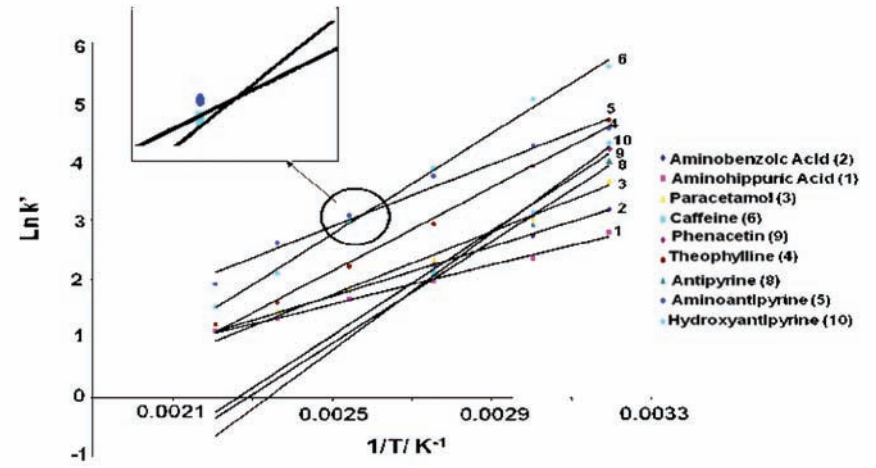

Figure 1: Van't Hoff plot of a series of polar compounds for a range of temperatures with a mobile phase of 0.1% formic acid (aq), flow rate 0.3 ml/min, injection volume 10 µl.

Results and discussion

The initial work investigated the selectivity differences observed with a series of compounds eluting from a column at differing temperatures. Figure 1 shows a plot of the 1/T vs. Ln k'. The relationship between the capacity factor and the temperature is well defined and derives from the Van't Hofft equation. This relationship is based on some assumptions, primarily that the interaction between the stationary phase and the analyte does not alter, and that the temperature merely affects the rate of adsorption and desorption within the column. Clearly, over a wide temperature range it might be expected that

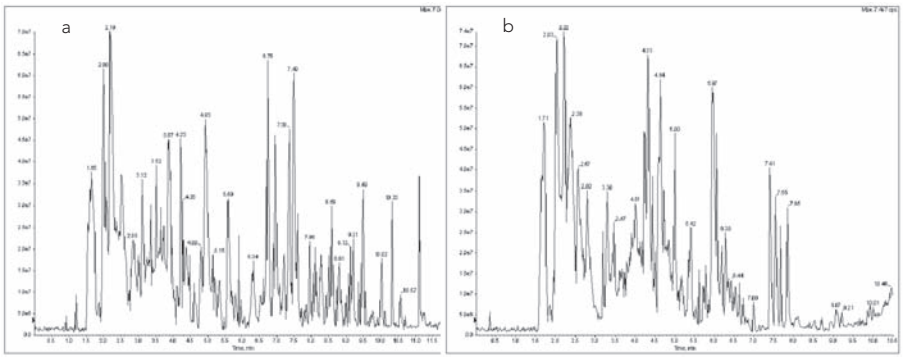

Figure 2: Mass Chromatogram (TIC) obtained from the LC-MS analysis of rat urine in positive ESI.
A: elevated temperature analysis with a combined thermal and flow rate gradient, The thermal gradient started at isothermal conditions for 2 minutes at 50 °C, followed by a steep gradient (over 6 min) to the maximum applied temperature of 180 °C, where it was held for 1 minute before the re-equilibration step to the initial temperature (10 min). The "required" pressure was fixed at 9000 psi, and a model was used to derive flow rates, thus at the beginning of the run the flow rate was 0.25 ml/min, but at the end it was 0.54 ml/min
B: conventional acetonitrile gradient in isothermal conditions (58 °C). Initially and till 0.5 min acetonitrile content 0%, then linear increase to 20% acetonitrile at 4 min, then linearly to 95% acetonitrile at 9 min where it was held isocratically for 1 min; finally an equilibration step for 3 min (0% Acetonitrile). The flow rate was 0.25 ml/min.

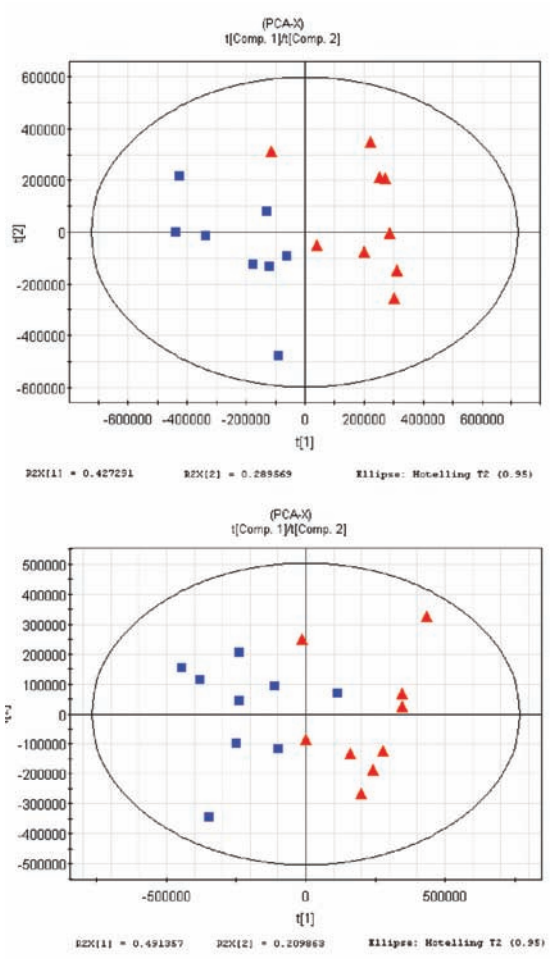

Figure 3: PCA scores plots (pc1 vs pc2) generated in SIMCA P, from the two data sets. A: thermal gradient in HPLC with (+ve) ESI detection; B: conventional RP-LC gradient with (+ve) ESI detection; Blue boxes represent lean rats; red triangles represent fat rats.

the surface morphology might change for some materials and that this in itself would result in different types of interactions between the analyte and the stationary phase.

It can be seen from Figure 1 that the relationship between 1/T and Ln k' is linear, suggesting that the mode of interaction between the analyte and the stationary phase is consistent across the temperature range under investigation. It can also be seen from Figure 1 that some experimental data points are missing. It was not possible to detect the peaks eluting at these elevated temperatures and it was assumed that for hydroxyantipyrine, antipyrine, and phenacetin, there was some form of thermal degradation occurring in the column.

One interesting phenomenon that is highlighted in Figure 1 is that the elution order for caffeine and aminoantipyrine alters. Thus at temperatures below 103°C, the elution order for the pair is aminoantipyrine followed by caffeine, whereas above this critical temperature the elution order is reversed.

One of the limitations of using an isothermal separation is that the run time will be dependant on the elution time of the first set of peaks and also the last. If the temperature is too high then there will not be enough separation of the early eluters, whereas too low a temperature will result in the late eluting components of the test mixture eluting at a prohibitively long retention time.

Figure 2 [7] demonstrates how high temperature liquid chromatography (HTLC) can be applied to the analysis of very complex mixtures. In this case, a metabolomic sample (rat urine) has been analysed using a conventional binary mobile phase gradient system, and also using an isocratic system with a thermal gradient to optimise the separation. The data from this experiment proved to be very useful, as there are substantial selectivity differences between methanol and water, even at elevated temperatures where water behaves more like a lipophillic mobile phase. The differences in selectivity are incredibly useful in metabolomic studies which are inherently looking for information hidden in a raft of data. The use of two potentially orthogonal separation techniques allows for the elucidation of this data in a much easier format. This is demonstrated in the principal component analysis (PCA) plots shown in Figure 3 [7].

These were obtained from both the binary solvent chromatographic system and also from the isocratic, thermal gradient chromatographic system. In both approaches a clear discrimination between the two sample sets (lean and fat rats) is obtained using the PCA analysis. Although both approaches are able to separate between the two data sets, the data that is used to separate the two data sets is not the same allowing the separation scientist to unravel further information about the metabolome.

The final application is based around the use of Hypercarb™ (Porous graphitic carbon, PGC). This is a unique chromatographic material made purely from carbon, which results in a highly retentive stationary phase. The manufacture of PGC involves a range of steps with varying degrees of extreme conditions from concentrated sodium hydroxide to temperatures in excess of 2000 K. This results in a stationary phase that it very inert and very stable to a wide range of chemical environments. In particular the use of elevated temperatures is ideally suited to this material as it can readily withstand very extreme temperatures beyond the temperatures used even in high temperature GC. One of the limitations of this material is that because of the amount of carbon present it is very hydrophobic, resulting in a highly retentive material. At low temperatures this can present a challenge as elution of hydrophobic analytes can be challenging, however as the temperature is increased so the elutropic strength of the mobile phase will increase resulting in a reduced retention. Unlike silica stationary phases, carbon does not have any active moieties that are

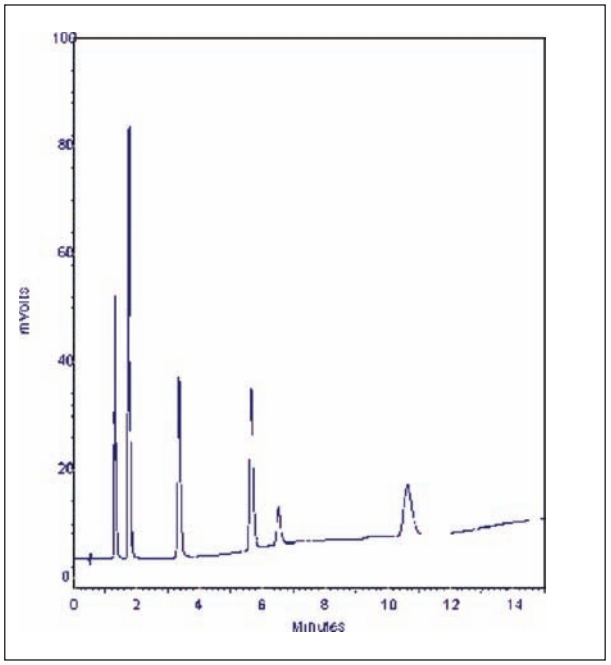

Figure 4 : Column: Hypercarb™ 5 µm, 100 x 4.6mm, mobile phase: H2O, flow rate: 2.0 ml/min
Temp. gradient: 150 to 200°C @15 °C/min, hold at 200°C, detection: UV @ 254 nm
Analytes in order of elution; Cytosine, Uracil, Thymine, Hypoxanthine, Guanine, Xanthine

susceptible to thermal degradation at extremes of temperature and as a result it is more inert.

Figure 4 shows a chromatogram obtained using this alternative experimental arrangement. A thermal gradient is used to elute the series of purines and pyrimidines from the column, and it can be seen that all of the series are eluted from the column with good resolution within twelve minutes. The use of the thermal gradient reduces the overall retention time of the last eluting peak and also means that a purely aqueous mobile phase can be employed. The other advantage of running at the elevated temperatures is that a higher flow rate can be utilised, however for this to be of benefit the detector has to be capable of coping with the specified flow rate.

Conclusions

It has been demonstrated that the use of elevated temperatures within the field of liquid chromatography has substantial benefits. The importance of characterising the physical parameters of the experimental setup was discussed. It can be seen that the relationship that exists between the pressure, temperature and the flow within the column can be readily utilised to ensure that a separation can be achieved on a column using elevated temperatures which would not be feasible at a lower temperature due to the physical constraints of the pumping systems. It should be noted however that there are some disadvantages of using this approach when employing sub 2 micron material, namely that there can be a loss of efficiency dependant on the flow that is employed. Examples were then given of temperature gradients and the advantages that using these can give the separation scientist. The use of isobaric thermal gradients is an interesting concept and one that manufacturers of pumps could develop to allow simple optimisation of a separation.

Hypercarb is clearly seeing renewed interest as the general interest in this type of technology increases. This is a unique stationary phase that is ideally suited to the use within HTLC. Its ability to naturally withstand the elevated temperatures, and be chemically inert make it a very robust phase. At low temperatures it can suffer because of the degree of hydrophobicity, however at elevated temperatures even non polar compounds can be eluted from it.

So where will this technology go? The advent of thermally stable stationary phases has demonstrated that there are applications that HTLC is suited to. However, for further acceptance of this technology there has to be a step change in the mind set of the separation scientist, to accept that not all compounds are thermally labile and that clever utilisation of thermal gradients will result in a thermally stable HPLC system. It is certain that whatever developments happen, it will always be a hot topic of conversation.

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