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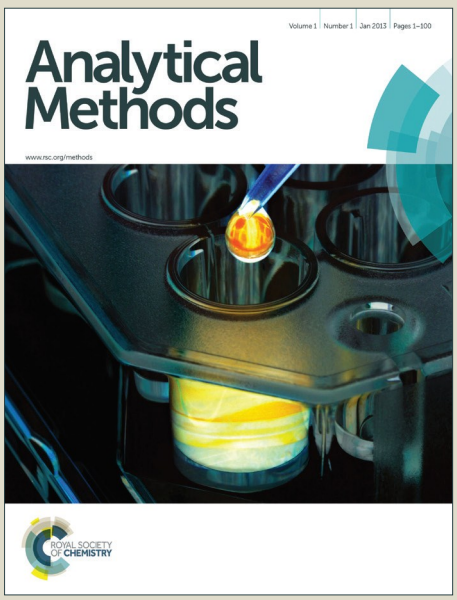

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Overcoming solvent mismatch limitations in 2D-HPLC with temperature programming of isocratic mobile phases

Brendan J. Holland, Xavier A. Conlan, Paul S. Francis, Neil W. Barnett

and Paul G. Stevenson*

Deakin University, Geelong, Australia. School of Life and Environmental Sciences, Centre for Chemistry and Biotechnology

* Corresponding author:

Dr Paul G Stevenson

Deakin University, School of Life and Environmental Sciences,

Locked Bag 20000, Geelong, Vic 3220, Australia.

Tel: +61 3 5227 2241

E-mail: paul.stevenson@deakin.edu.au

Abstract

This work describes a method for two-dimensional high performance liquid chromatography (2D-HPLC) that uses an isocratic mobile phase with a temperature gradient in the first dimension. Temperature programming was used to manipulate solvent elution strength in place of a mobile phase concentration gradient. This ensured that all eluent fractions transferred into the second dimension were of an identical solvent composition, i.e. the second dimension injection solvent did not increase during the course of the analysis. When applied to a complex natural product extract of coffee, the separation was completed in 35 min and had an orthogonality of 35% (calculated using the bins method) and a spreading angle of 52° as determined *via* a geometric approach to factor analysis. This approach, incorporating a temperature gradient in the first dimension, compared favourably to previously reported 2D-HPLC separations of coffee, with similar or shorter analysis times.

Keywords: Two-dimensional high performance liquid chromatography (2D-HPLC); temperature programming; solvent strength mismatch; coffee

1. Introduction

Maximising peak capacity is a significant goal in the development of two-dimensional high performance liquid chromatography (2D-HPLC) of chemically complex samples.[1] High peak capacities have been achieved by coupling columns of different retention mechanisms to ensure the entire separation space is used; however, the most divergent HPLC separations (such as coupling reversed and normal phase systems) are naturally incompatible due to solvent immiscibility.[1-3] Researchers have used various strategies to couple mismatched HPLC mobile phases, but they have inherent limitations such as a requirement for specialised equipment, excessive time to evaporate the eluent solvent and re-dissolve in a chemically appropriate solution, and/or decreased resolution and sensitivity.[4-7]

Reversed phase (RP) × RP separations do not have the same immiscibility problems but are not immune to adverse chemical difference between dimensions, including solvent breakthrough, and pH, solvation and viscosity differences, which are often neglected.[8-13] The effects of mismatch between the injection solvent and initial mobile phase have been extensively reported and are of particular importance in 2D-HPLC when the eluent composition is changing (as in the case of gradient elution) or when performing a RP × HILIC separation.[14-17] In these instances, the solvent mismatch typically leads to zone broadening or split peaks, a phenomenon caused by a slow rate of solute adsorption onto the stationary phase from the solvent plug after injection.[14-19]

The application of mixed-mode stationary phases is advantageous to increase the peak capacity in gradient RP × RP 2D-HPLC, combining unique mechanisms of retention in the first dimension. However, this approach is susceptible to the limitations of solvent mismatch (from the addition of ionic solutes to the organic eluent that are ultimately transferred to the second dimension).[20]

To overcome the difficulties experienced with solvent mismatch, the first dimension can be run in isocratic mode, which allows for transfer of a consistent injection plug concentration and the use of a full gradient in the second dimension. Unfortunately, this leads to longer elution times and inefficient utilisation of separation space.[18, 21] Researchers have attempted to overcome these limitations by: using a gradient in the first dimension and modifying the eluent with a weak solvent;[9, 22] using a column with larger inner diameter in the second dimension;[9] or using small fraction volumes. These approaches are unsuitable for use with the fast second dimension gradients required for online 2D-HPLC.[22]

With recent commercial developments in 2D-HPLC, shift gradients have been reported that involve a relatively shallow change in the initial solvent composition of the second dimension, with each successive injection ensuring that the plug being transferred between the two columns is closer to the starting mobile phase concentration.[23, 24] This protocol combines the benefits of full and parallel gradients with shorter re-equilibration time, but does so at the expense of overall peak capacity.[24]

Stevenson and co-workers[16] have described an alternative stratagem coupling HILIC and RP-HPLC for the separation of a coffee extract, which employed a counter gradient that was merged with eluent fractions as they were collected to artificially control the second dimension injection solvent.[16, 25] This counter gradient gave a 2D-HPLC separation of coffee that occupied 71% of the space from a previously indecipherable retention profile.[16] and was also successfully used in a RP × RP 2D-HPLC analysis of human urine.[25]

This paper explores the potential of a temperature programmed gradient in first dimension of 2D-HPLC. Although temperature programming is extensively used in gas chromatography, its application in HPLC is limited due to heat-transfer restrictions of the relatively wide-bore columns.[26, 27] Aqueous solvents are known to exhibit less polar characteristics as they are heated and therefore, if used under isocratic conditions,

temperature programming can provide the benefits of gradient chromatography in manipulating retention, whilst maintaining a constant mobile phase eluent.[28] Accordingly, peak focusing in the second dimension injection process can be maximised if temperature programming is used in the first dimension with a low organic solvent concentration. The solvent composition of the first dimension is not changing, so the initial mobile phase of the second dimension can be optimised for the entire separation. The full benefits of gradient elution are then realised in both dimensions without deterioration in peak shape.[26, 29] Temperature programmed separations are performed using the standard configurations of modern HPLC systems and the instrument control timetable that make them easy to implement and the associated reduction of organic solvent consumption makes these separations cost effective and environmentally friendly.[30]

2. Experimental

Chemicals, reagents and preparation of standards

Analytical grade methanol was obtained from Chem Supply (Gilman, SA, Australia) and HPLC grade from Ajax (Tarren Point, NSW, Australia). Reagent grade toluene, ethyl-, *n*-propyl-, butyl- and pentyl benzene, bisphenol A, caffeine, phenol, anisole and resorcinol were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Thiourea was supplied by BDH chemicals (Frenchs Forest, NSW, Australia). Dopamine hydrochloride and homovanillic acid were sourced from Alfa Aesar (Gymea, NSW, Australia), and morphine from GlaxoSmithKline (Port Fairy, Vic., Australia). Deionised water filtered through a 0.45 µm filter (Sigma-Aldrich) before use in the mobile phase.

Sample preparation

Fresh coffee samples were obtained daily *via* extraction of 5 g Ristretto brand Nespresso coffee beans (Nespresso, North Sydney, NSW, Australia) with 30 mL hot water, using a Saeco coffee machine (Royal Pro model). The extract was filtered with a 0.45 µm syringe filter and prepared with a concentration of 5% methanol prior to analysis.

Morphine (1×10^{-3} M) was prepared by sonication in acidified deionised water to aid dissolution and diluted to 1×10^{-5} M for analysis. Bisphenol A, phenol, anisole, caffeine, propyl benzene, butyl benzene and n-pentyl benzene (10 mg L^{-1}) were prepared in methanol and diluted to 1×10^{-5} M at the relevant initial methanol composition of the first dimension for analysis. The remaining analytes were prepared in deionised water at 10 mg L^{-1} and diluted to 1×10^{-5} M in methanol matching the starting composition of the method.

High performance liquid chromatography

All analyses were completed with an Agilent 1260 chromatography system consisting of a binary capillary pump with solvent degasser, 1290 Infinity binary pump with solvent

degasser, autosampler, 1290 Infinity thermostatted column compartment containing an in-built 8-port, 2-position switching valve with two 40 µL sample loops, and a diode array detector module that monitored absorbance at 254 nm (Agilent Technologies, Mulgrave, Vic, Australia). Agilent Chemstation software was used for system control and data acquisition. An online comprehensive two-dimensional separation was developed and individually adapted for the standards and sample. At the separation interface sequential 1 min fractions (standards) or 2 min fractions (coffee) were collected using the switching valve and two sample loops, in a configuration where one loop fills whilst the other is emptying into the second dimension. A Gemini C18 column (50 × 1 mm column dimensions, 3 µm particle diameter, 110 Å pore diameter, Phenomenex, Lane Cove, NSW, Australia) was used in the first dimension. A volume of 40 µL was injected by the autosampler into a mobile phase flowing at 20 µL min^{-1}, containing a methanol concentration of 5% for the sample and 40% for standards.

Temperature programming was performed in the first dimension where the column temperature was increased from 15 °C to 91.8 °C at a rate of 3.2 °C min^{-1} immediately upon injection, then held isothermally at 91.8 °C until the end of the separation. Separations in the second dimension were carried out using a Kinetex Phenyl-Hexyl column (100 × 4.6 mm column dimensions, 2.6 µm particle diameter, 110 Å pore diameter, Phenomenex) isothermally at 60 °C with a flow rate of 4.5 mL min^{-1} (standards) or 4.0 mL min^{-1} (coffee). For the standards a methanol gradient was used, increasing linearly from 67% to 100% over 0.2 min before being held for a further 0.2 min at 100%, with a 0.6 min re-equilibration time. A linear gradient from 5% to 100% methanol was used for the coffee extract, carried out over 1.4 min with a 0.6 min re-equilibration time. To allow even cooling of the first dimension column a thermal re-equilibration time of 45 min was used between injections.

Orthogonality, *O*, was calculated using the bins method, where the separation space is divided into a given number of bins (Σbins) equal to the number of peaks.[31] P_{max} was then calculated by totalling the area of all normalised bins containing peaks.[3] Calculating the fraction surface coverage using this method was considered useful by Gilar and co-workers[31] after undertaking a review of the current popular methods.

3. Results and Discussion

3.1. Operational parameters for temperature programmed 2D-HPLC

A Gemini C18 column was selected for the first dimension separation, as the internal diameter of 1 mm allows for a more uniform heating than typical analytical scale formats.[26] Although narrower bore columns with an internal diameter of 0.5 mm allow for a more rapid transfer of thermal energy, they were only rated to operate up to 60 °C. The column chosen was suitable for use at temperatures up to 90 °C, according to the manufacturer. This extended operational range is a significant factor for method development considering that temperature has a smaller relative influence on manipulating retention than changing the mobile phase.[26] The instrument design included a thermostat with a mobile phase preheater prior to the column which is considered beneficial in reducing axial temperature gradients, and a capillary HPLC pump to ensure steady delivery of the required low flow rate.[30] The thermal equilibration time between subsequent analysis was measured by adding duration taken for the thermostat to return to 15 °C (from 90 °C) and the time required for even cooling of the column. The latter value was determined by injecting thiourea, phenol and anisole after the thermostat had cooled and a specified period had lapsed (see Figure 1). Reproducible retention of these analytes was obtained after a column thermal equilibration period of 17.5 min. Cooling time for the thermostat is measured in the next section.

3.2. Optimising the temperature gradient rate

The temperature limits for the method were established by examining the heating, cooling and re-equilibration rates for the thermostat and column system. A gradient starting temperature of 15 °C and maximum of 90 °C was selected based on manufacturer specifications. Based on empirical investigations (not shown) the column thermostat was capable of heating over the temperature range in 13 min at an average rate of 5.8 °C min^{-1}, and cooled over 20 min at 3.8 °C min^{-1}. The HPLC column compartment was not designed to

operate a thermal gradient and step gradients were used to mimic a linear temperature change. However, a detailed reproducibility study was not performed for this aspect of the investigation. The interval required between injections was almost 40 min, made up of the 17.5 min equilibration time previously determined and a duration of 20 min for cooling of the thermostat. This interval of 40 min is long compared to 12 min required to equilibrate a mobile phase and is due in part to the slow process of energy transfer from the heating block to the column *via* the air in the oven (3.8 °C min^{-1}, as measured by the instrument thermostat). Although beyond the scope of this study, integrating a cooling system or using a temperature source in direct contact with the column could reduce the re-equilibration time and render the method more suitable for high throughput routine analysis.[30] Conversely, the controlled column heating gives the method greater power to separate closely eluting compounds by gradually manipulating their retention factors, whilst maintaining acceptable retention times for more strongly retained species.

Linear temperature gradient rates of 5 and 8 °C min^{-1} over the operating range were studied for their effect on the retention and separation of selected polar and non-polar standards. These temperature gradient rates were selected because they fell within the operational capabilities of the thermostat. For simplicity, non-linear thermal gradients were excluded from the study. A mobile phase composition of 40% methanol was selected to reduce the retention times of the non-polar analytes while maintaining an adequate resolution of the earlier eluting compounds, which was lost at higher methanol concentrations. For applications involving the separation of more polar compounds the mobile phase composition can be lowered to provide further resolution as required. The retention of a series of standards when separated under various isothermal and gradient temperature profiles are presented in Table 1. The application of a thermal gradient with an isocratic mobile phase composition demonstrated benefits in both improved retention of the polar analytes at low temperatures

and shorter elution times for the non-polar analytes at high temperatures. The significance of even temperature programming was seen in the reduction in elution times of butyl and n-pentyl benzene from approximately three hours under isothermal conditions of 50 °C to less than an hour at a gradient of 5 °C min^{-1}. When comparing the retention data at 50 °C isothermal and an increase of 5 °C min^{-1}, the temperature gradient also maintained (and in some cases improved) the separation of these standards. The selectivity factor ($\alpha = k_2/k_1$) of adjacent peaks suggests that a lower initial temperature allowed for better resolution of poorly retained components, but at higher temperatures the relative retention was much lower while still achieving baseline separation. The utilisation of temperature to influence selectivity is highlighted by the changing retention order of both L-alanine and bisphenol A. From the data presented in Table 1, an 8 °C min^{-1} thermal gradient was selected for the first dimension separation to balance resolution and retention behaviour, and maintain a short analysis time.

3.3. Developing a two-dimensional method for separation of the standard mixture

In the online comprehensive mode of 2D-HPLC, the sampling frequency is limited by the second dimension, which must complete its separation and re-equilibration cycle in the time taken for the first to fill the sample loop. A fast second dimension separation was required with a mobile phase gradient commencing with an ideal minimum of 40% methanol to avoid potential mismatch issues with the first dimension. After comparing void volume and selectivity of a number of columns the phenyl-hexyl column was selected to develop a fast second dimension cycle with an aggregate time of 1 min, comprising of 0.4 min separation and 0.6 min re-equilibration periods. The final second dimension method used a 68% to 100% methanol gradient, in order for the separation and re-equilibration cycle to be completed in 1 min. The first dimension flow rate was adjusted to allow the desired sampling rate of 3 cuts per peak, at a frequency of 1 fraction per minute as dictated by the second

dimension. This was achieved by reducing the flow rate from 50 to 20 μL min^{-1} and scaling the temperature gradient down from 8 to 3.2 °C min^{-1}.

The separation dimensions were coupled *via* a switching valve and sample loops to create a comprehensive on-line 2D-HPLC analysis of the 14 standards. The resulting chromatogram is illustrated in Figure 2. Although 120 min was required to elute all standards, peaks for *n*-propyl, butyl- and pentyl-benzene were not observed due to a loss in sensitivity resulting from significant band broadening in the first dimension and dilution during the transfer of eluent between dimensions. Note multiple peak maxima are observed for two of the earlier eluting standards, giving three more maxima than expected. Although the resulting 2D-HPLC is separation is highly correlated and not ideal chromatography, standards which co-eluted in the first dimension were separated in the second. Symmetric peak shapes were also observed for all fractions owing to the consistent eluent concentrations injected into second dimension. Whilst correlation between the dimensions could be reduced with a more rigorous approach to selecting the stationary phases, at least three hours of analysis time was saved by incorporating a temperature gradient.

3.4. Adaption of the two dimensional method for a real sample separation

A separation of coffee extract was designed to assess the performance of a temperature gradient on a real, chemically complex sample. The methanol composition of the first dimension mobile phase was reduced to 5% to facilitate better retention and separation of the polar components within the sample, and the temperature gradient rate was unaltered at 3.2 °C min^{-1} with a 20 μL min^{-1} flow rate. The second dimension was also modified to improve the method sensitivity for the coffee by doubling the fraction volumes collected from the first. Although this reduced the sampling rate (to 1.5 fractions per peak), greater separation in the second dimension was achieved by lowering the flow and using a larger difference between the initial and final gradient compositions. Representative chromatograms

of the separation behaviour in each dimension are shown in Figure 3 and the resulting 2D-HPLC separation of the coffee extract is illustrated in Figure 4. With the aid of peak detection algorithms a total of 34 peaks were detected in an analysis time of 35 min. This separation provided an orthogonality of 35% as calculated *via* the bins method and a spreading angle of 52° determined by a geometric approach to factor analysis.[3, 32, 33]

Both the orthogonality and spreading angles compare favourably against previously reported 2D-HPLC separations of coffee. For the same sample matrix Bassanese and co-workers reported an orthogonality of 39% with a 48 min separation.[25] Stevenson *et al.* incorporated a counter gradient in between dimensions and reported a 10% usage of separation space and 70.5° spreading angle over a 5.25 hour analysis.[16] Mnatsakanyan *et al.* reported a spreading angle of 56° with a run time of 21 hours.[34] Two-dimensional HPLC using a temperature gradient in the first dimension provided a more favourable analysis time than those of the latter two studies,[16, 34] even when considering the long re-equilibration between analyses. However, the use of a narrow bore HPLC column resulted in a lower sensitivity whereby fewer peaks were detected than other methods. This arose from the combined small injection volumes on the narrow bore column and transfer of fractions between separation dimensions.

Although this is a preliminary investigation, the use of a temperature gradient shows merit in the first dimension to ease solvent mismatch issues commonly associated with mobile phase gradient based 2D-HPLC. Column thermostats for HPLC instrumentation is not designed to operate a gradient, for a more thorough study a new method to precisely control the separation temperature and transfer this energy to the column must be designed.

4. Conclusions

Temperature programming is a valuable tool for 2D-HPLC and suited for applications that demand fast analysis times and high peak capacity; the deleterious effects of solvent mismatch and reduced sensitivity from other complex interfacing solutions are avoided. An online comprehensive 2D-HPLC method with a temperature programmed first dimension separation coupled to a mobile phase gradient in the second was successfully applied to analysis of a selection of standards and a coffee extract. The use of temperature programming avoided solvent incompatibility at the separation interface, ensuring peaks in the second dimension retained a symmetrical profile which led to an improved peak capacity and reduced retention times.

However, the need for samples and HPLC columns stable at high temperatures is limiting for many applications. The extended thermal re-equilibration times in the order of 40 min are also unfavourable for high throughput routine analysis, requiring further attention from the instrumental and column design viewpoint. The analysis time however was still more favourable than other methods described in the literature. A coffee extract was successfully separated using the developed method which maintained the separation performance observed in other separations.

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References

1. G. Guiochon, N. Marchetti, K. Mriziq and R. A. Shalliker, *Journal of Chromatography A*, 2008, **1189**, 109-168.
2. J. C. Giddings, *Journal of Chromatography A*, 1995, **703**, 3-15.
3. M. Gilar, P. Olivova, A. E. Daly and J. C. Gebler, *Analytical Chemistry*, 2005, **77**, 6426-6434.
4. Y. Wei, T. Lan, T. Tang, L. Zhang, F. Wang, T. Li, Y. Du and W. Zhang, *Journal of Chromatography A*, 2009, **1216**, 7466-7471.
5. F. Cacciola, M. Beccaria, P. Donato, L. Mondello and P. Dugo, *LC GC Eur.*, 2014, **27**, 570-577.
6. J. Haun, T. Teutenberg and T. C. Schmidt, *Journal of Separation Science*, 2012, **35**, 1723-1730.
7. W. J. Sonnefeld, W. H. Zoller, W. E. May and S. A. Wise, *Analytical Chemistry*, 1982, **54**, 723-727.
8. R. A. Shalliker and G. Guiochon, *Journal of Chromatography A*, 2009, **1216**, 787-793.
9. J. Layne, T. Farcas, I. Rustamov and F. Ahmed, *Journal of Chromatography A*, 2001, **913**, 233-242.
10. W. Naidong, Y.-L. Chen, W. Shou and X. Jiang, *Journal of Pharmaceutical and Biomedical Analysis*, 2001, **26**, 753-767.
11. R. A. Shalliker and G. Guiochon, *Analyst*, 2010, **135**, 222-229.
12. N. E. Hoffman, S.-L. Pan and A. M. Rustum, *Journal of Chromatography A*, 1989, **465**, 189-200.
13. D. R. Stoll, K. O'Neill and D. C. Harmes, *Journal of Chromatography A*, 2015, **1383**, 25-34.
14. P. Jandera, J. Fischer, H. Lahovská, K. Novotná, P. Česla and L. Kolářová, *Journal of Chromatography A*, 2006, **1119**, 3-10.
15. K. Horváth, J. N. Fairchild and G. Guiochon, *Journal of Chromatography A*, 2009, **1216**, 7785-7792.
16. P. G. Stevenson, D. N. Bassanese, X. A. Conlan and N. W. Barnett, *Journal of Chromatography A*, 2014, **1337**, 147-154.
17. B. J. VanMiddlesworth and J. G. Dorsey, *Journal of Chromatography A*, 2012, **1236**, 77-89.
18. P. Jandera, *Journal of Chromatography A*, 2012, **1255**, 112-129.
19. G. Vivó-Truyols, S. van der Wal and P. J. Schoenmakers, *Analytical Chemistry*, 2010, **82**, 8525-8536.
20. D. Li, R. Dück and O. J. Schmitz, *Journal of Chromatography A*, 2014, **1358**, 128-135.
21. D. R. Stoll, J. D. Cohen and P. W. Carr, *Journal of Chromatography A*, 2006, **1122**, 123-137.
22. D. Stoll, E. Talus, D. Harmes and K. Zhang, *Anal Bioanal Chem*, 2015, **407**, 265-277.
23. F. Bedani, W. T. Kok and H.-G. Janssen, *Analytica Chimica Acta*, 2009, **654**, 77-84.
24. D. Li and O. Schmitz, *Anal Bioanal Chem*, 2013, **405**, 6511-6517.
25. D. N. Bassanese, B. J. Holland, X. A. Conlan, P. S. Francis, N. W. Barnett and P. G. Stevenson, *Talanta*, 2015, **134**, 402-408.
26. M. H. Chen and C. Horváth, *Journal of Chromatography A*, 1997, **788**, 51-61.
27. J. Clark, in *Technology & Tools*, American Chemical Society, 2004, vol. August 2004.
28. J. W. Dolan, *Journal of Chromatography A*, 2002, **965**, 195-205.

29. N. M. Djordjevic, F. Houdiere, P. Fowler and F. Natt, *Analytical Chemistry*, 1998, **70**, 1921-1925.
30. N. M. Djordjevic, P. W. J. Fowler and F. Houdiere, *Journal of Microcolumn Separations*, 1999, **11**, 403-413.
31. M. Gilar, J. Fridrich, M. R. Schure and A. Jaworski, *Analytical Chemistry*, 2012, **84**, 8722-8732.
32. P. G. Stevenson, M. Mnatsakanyan, G. Guiochon and R. A. Shalliker, *Analyst*, 2010, **135**, 1541-1550.
33. Z. Liu, D. G. Patterson and M. L. Lee, *Analytical Chemistry*, 1995, **67**, 3840-3845.
34. M. Mnatsakanyan, P. G. Stevenson, X. A. Conlan, P. S. Francis, T. A. Goodie, G. P. McDermott, N. W. Barnett and R. A. Shalliker, *Talanta*, 2010, **82**, 1358-1363.

Tables

Table 1. Comparison of retention factor (k) and retention time (t_R) over a selection of isothermal and temperature programmed trials, using an isocratic 40% methanol mobile phase. *Data not collected due to excessive analysis time

Analyte	15 °C isothermal		50 °C isothermal		85 °C isothermal		5 °C min^{-1}	8 °C min^{-1}
	t_R (min)	k	t_R (min)	k	t_R (min)	k	t_R (min)	t_R (min)
dopamine	1.19	0.1	1.10	0.1	1.04	0.0	1.15	1.21
morphine	1.19	0.1	1.10	0.1	1.04	0.0	1.15	1.19
thiourea	1.28	0.2	1.20	0.2	1.17	0.1	1.26	1.29
resorcinol	1.70	0.5	1.41	0.4	1.27	0.3	1.63	1.69
caffeine	1.79	0.6	1.49	0.5	1.35	0.3	1.76	1.95
L-alanine	1.97	0.8	1.80	0.8	1.62	0.6	1.89	2.05
homovanillic acid	2.16	1.0	1.55	0.5	1.35	0.3	2.08	2.19
phenol	2.59	1.4	1.84	0.8	1.53	0.5	2.44	2.58
bisphenol A	5.56	4.1	6.87	5.7	3.02	2.0	-	
anisole	6.91	5.3	3.79	2.7	2.60	1.6	6.19	2.56
toluene	14.26	10.9	7.13	6.0	4.26	2.6	11.37	10.95
ethyl benzene	30.92	24.8	13.32	12.1	6.96	4.8	18.10	17.26
n-propyl benzene	79.2	65.0	28.5	26.9	12.8	9.7	27.0	26.56
butyl benzene	208.2	172.5	63.20	61.0	24.5	19.4	39.0	39.9
pentyl benzene	>350	*	139.99	136.3	48.0	39.0	58.5	62.2

Figures

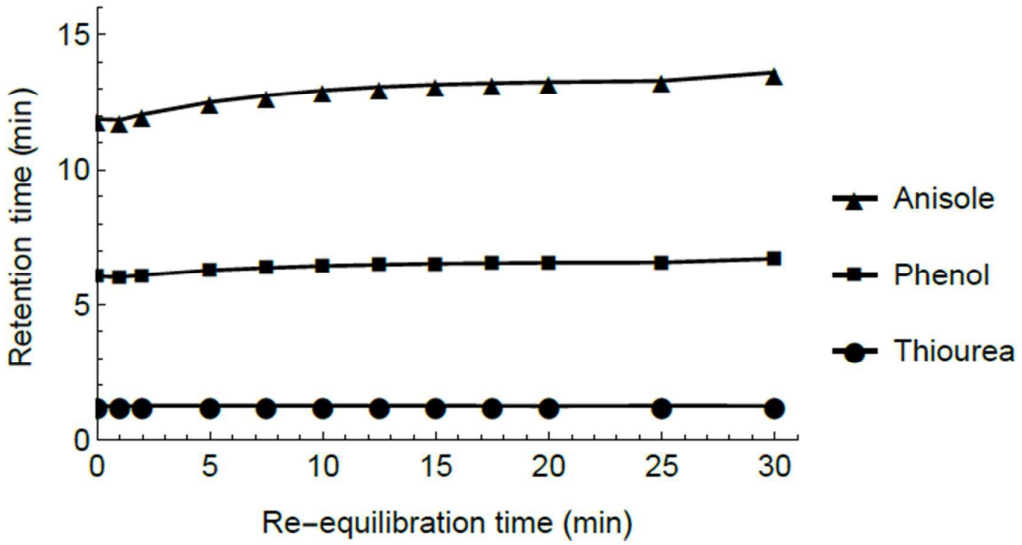


Figure 1. Influence of thermal re-equilibration time on the retention behaviour of three analytes, thiourea, phenol and anisole. Re-equilibration time began after the thermostat returned to the initial temperature of 15 °C.

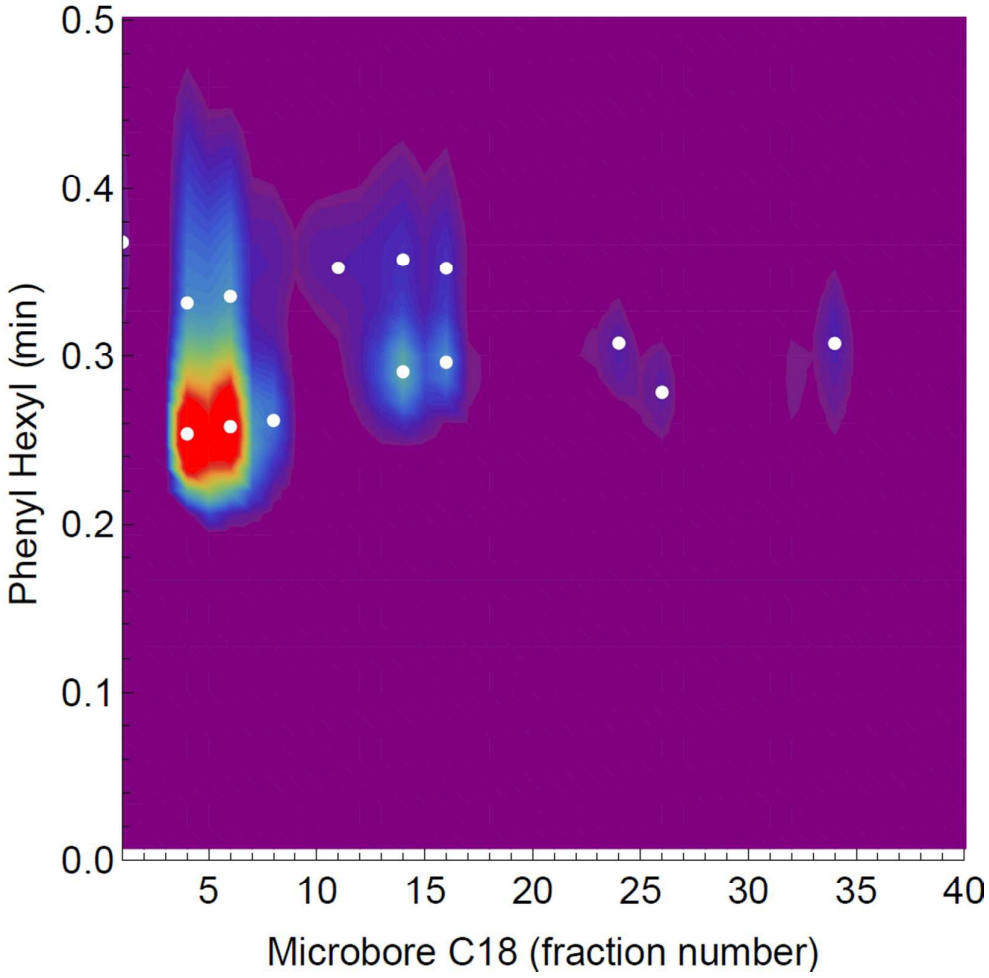
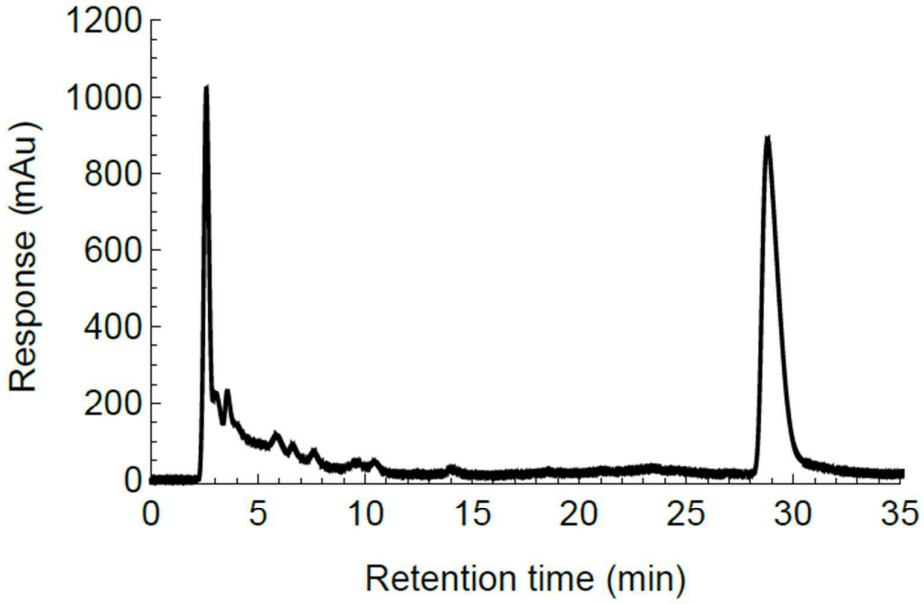


Figure 2. Coupled online two-dimensional separation of the standard mix, where white dots represent peaks picked using algorithms.[32] Although the separation time of the first dimension is 120 min, the later eluting *n*-propyl-, butyl- and pentyl- benzene standards were not identified in the 2D chromatogram.

(a)



(b)

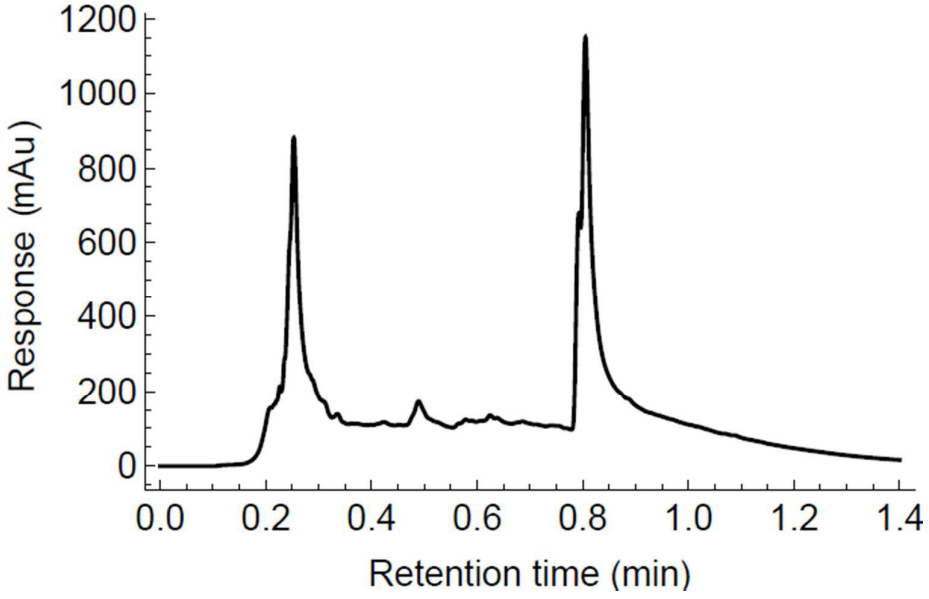


Figure 3. (a) First dimension separation of the coffee sample using a 3.2 °C min^{-1} gradient from 15 to 91.8 °C, with a mobile phase composition of 5% methanol. (b) Second dimension separation of coffee extract using the Kinetex column and a 1.4 min mobile phase gradient from 5% to 100% methanol, at a temperature of 60 °C.

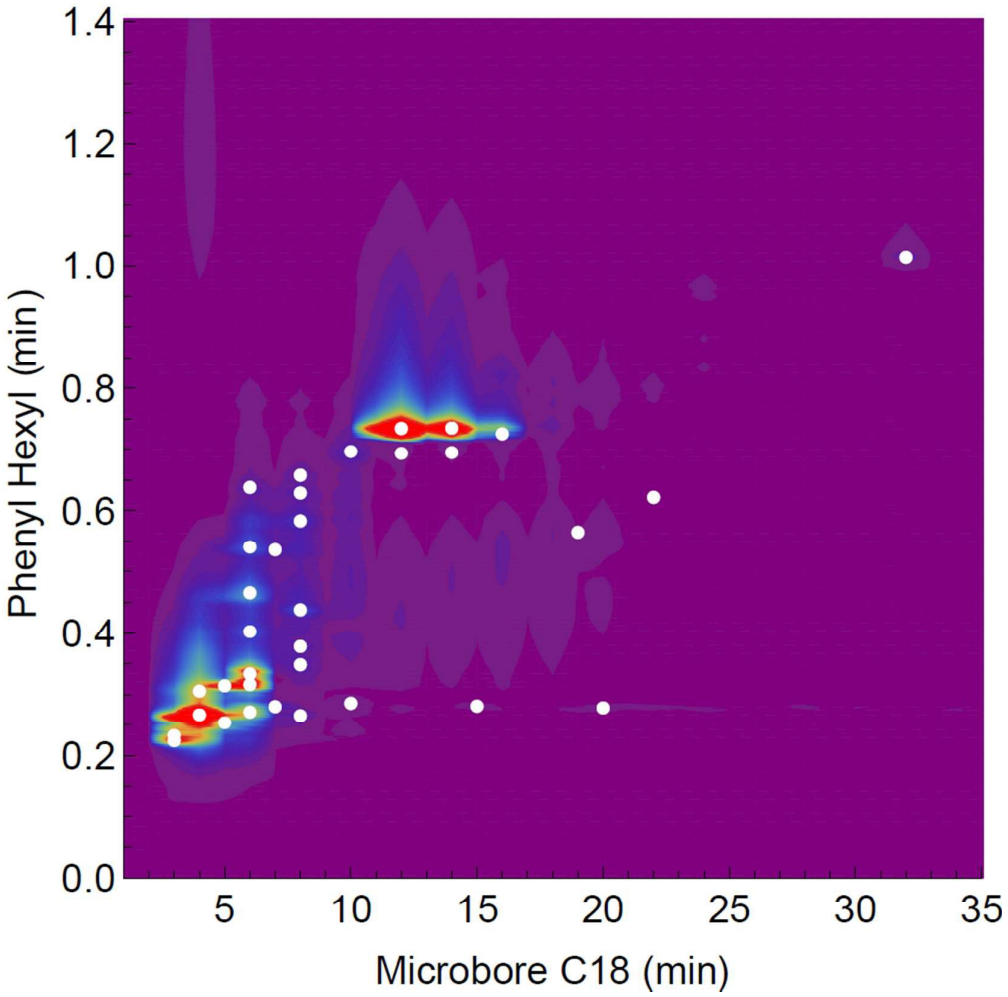


Figure 4. Two-dimensional separation of the coffee extract, where white dots represent peaks picked using algorithms, completed in 35 min.

Solvent incompatibility is a limiting factor when implementing 2D-HPLC. A programmed temperature gradient in the first dimension was developed to overcome this limitation by allowing an isocratic separation while maintaining selectivity.