Talanta 134 (2015) 402–408

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Protocols for finding the most orthogonal dimensions for two-dimensional high performance liquid chromatography

Danielle N. Bassanese [1], Brendan J. Holland [1], Xavier A. Conlan, Paul S. Francis, Neil W. Barnett, Paul G. Stevenson *

Centre for Chemistry and Biotechnology, School of Life and Environmental Sciences, Deakin University, Geelong, Vic. 3216, Australia

ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form
17 November 2014
Accepted 18 November 2014
Available online 26 November 2014

Keywords:
Two-dimensional high performance liquid chromatography
Orthogonality
Stationary phase optimisation
HPLC simulation
Potassium permanganate chemiluminescence
Bins method

ABSTRACT

The selection of two high performance liquid chromatography (HPLC) columns with vastly different retention mechanisms is vital for performing effective two-dimensional (2D-) HPLC. This paper reports on a systematic method to select a pair of HPLC columns that provide the most different separations for a given sample. This was completed with the aid of a HPLC simulator that predicted retention profiles on the basis of real experimental data, which is difficult when the contents of sample matrices are largely- or completely-unknown. Peaks from the same compounds must first be matched between chromatograms to compare the retention profiles and optimised 2D-HPLC column selection. In this work, two methods of matching peaks between chromatograms were explored and an optimal pair of chromatography columns was selected for 2D-HPLC. First, a series of 17 antioxidants were selected as an analogue for a coffee extract. The predicted orthogonality of the standards was 39%, according to the fractional surface coverage 'bins' method, which was close to the actual space utilisation of the standard mixture, 44%. Moreover, the orthogonality for the 2D-HPLC of coffee matched the predicted value of 38%. The second method employed a complex sample matrix of urine to optimise the column selections. Seven peaks were confidently matched between chromatograms by comparing relative peak areas of two detection strategies: UV absorbance and potassium permanganate chemiluminescence. It was found that the optimal combinations had an orthogonality of 35% while the actual value was closer to 30%.

1. Introduction

With the abundance of high performance liquid chromatographic columns currently available it can be confusing when selecting which stationary phase(s) to use [1]. To ensure the greatest possible separation power in two-dimensional high performance liquid chromatography (2D-HPLC) it is important to select two columns to reach maximal separation space utilisation [2–4]. Moreover, the selectivity of these columns can be further magnified by the experimental parameters under which the separation is completed, including the stationary and mobile phases [5], temperature [6] and pH [7]. However, great care must be taken when selecting these mobile phases as solvent-strength mismatch [8–10] and viscous fingering effects [11,12] can deform the HPLC peak.

Optimisation of 2D-HPLC dimensions requires that all experimental variables be compared against each other; however, an exhaustive approach is not practical as the retention times of common analytes must be known on a library of HPLC columns with different solvents/temperatures/pH, which can then be compared [3,13]. Column characterisation protocols are currently used by analysts to gain insight into selectivity [14]. The hydrophobic-subtraction model [15]–which accounts for column hydrophobicity, steric interactions, solute acidity, solute basicity, and capacity for cation exchange – is one protocol that has been adapted by chromatographers to select orthogonal columns for 2D-HPLC [16,17]. These protocols simplify the task of choosing columns by generating a simple visualisation scheme to easily contrast selectivities; however, methods for classifying columns are not always suitable for complex analysis of natural product extractions where there is little knowledge regarding the individual mechanisms of retention [18].

Typically, stationary phase selection for 2D-HPLC is completed following one of two protocols: use a series of standards to represent a more complicated sample matrix [19,20]; or use the sample itself to optimise the separation [21,22]. However, both of

* Correspondence to: Deakin University, Geelong Waurn Ponds Campus, 75 Pigdons Road, Waurn Ponds, Victoria 3216, Australia.
Tel.: +61 3 5227 2241; fax: +61 3 5227 2356.
E-mail address: paul.stevenson@deakin.edu.au (P.G. Stevenson).
[1] Co-first authors.

http://dx.doi.org/10.1016/j.talanta.2014.11.037
0039-9140/

these strategies are fundamentally difficult. Injecting a series of standards is both labour and solvent intensive, and, often, not enough information is known about the sample to adequately represent it. However, when the sample itself is used it is very difficult to find enough common peaks, with any degree of certainty, between several different chromatograms with common detectors such as UV absorbance [22].

Optimisation of HPLC experimental parameters has been assisted using appropriate simulation software [23], which is accurate for both isocratic and gradient elution modes. Optimisation requires a systematic strategy, which is time consuming [24,25], or a somewhat blind approach where trial-and-error separations are completed until the analyst reaches a satisfactory result [26]. Dolan and co-workers [23] compared the elution profile of simulated optimised chromatograms generated by *in-silico* optimisation software against real separations and found a very close agreement between the predicted and experimental data. This can be completed on any reversed phase HPLC system with the introduction of elution data from two gradient analyses into the software [23]. Importantly, on modern computers these simulations and predictions can be completed rapidly, saving hours of laboratory time and potentially thousands of dollars in solvent consumption [27].

In-silico optimisation extrapolates chromatograms by calculating key retention parameters for each peak. This requires a minimum of 2 injections per analyte if only the gradient time is to be optimised. If the separation temperature also needs optimisation then 4 injections per compound are needed. However, for the *in-silico* process to succeed, peaks must be matched in several chromatograms to generate the important retention parameters. This is a significant problem when selecting the most orthogonal (separation *via* different retention mechanisms) HPLC columns and solvents for 2D-HPLC [2,28], where significant changes in elution order can occur.

A robust method to measure the surface coverage of separations with varying numbers of components must be considered when calculating $f_{coverage}$. Gilar and co-workers [19] recently reviewed the current popular methods for calculating separation space utilisation. It was concluded that calculating the fraction surface coverage through a method defined by Gilar et al. [29] was useful for calculating the $f_{coverage}$ term of the 2D-HPLC peak capacity equation [19]. This approach divides the separation space by a given number of bins, Σbins, that is equal to the number of peaks; the area of all normalised bins containing peaks is then totalled giving P_{max} [29]. Orthogonality, O, was then calculated as a value between 0 and 1 according to Eq. (1) [29].

$$O = \frac{\sum \text{bins} - \sqrt{P_{max}}}{0.63 P_{max}} \quad (1)$$

The aim of this paper is to highlight the difficulties and problems associated with the two optimisation protocols: (1) using the sample to optimise the separation by comparing detection features with several detection strategies, and (2) using a series of standards to represent the complex mixture. Although all of the experimental conditions, including temperature and pH, will influence the selectivity of a separation the work presented here focuses on the problems associated with selecting HPLC columns and mobile phases for 2D-HPLC through the analysis of two different complex samples; the protocols presented can be extended to cover these other sources of selectivity.

2. Material and methods

2.1. Chemicals and reagents

Deionised water (Continental Water Systems, Victoria, Australia) was filtered through a 0.45 μm filter (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) before use. The organic modifiers used for this investigation included HPLC grade acetonitrile and methanol (Ajax Finechem Pty. Ltd., Taren Point, NSW, Australia). Trifluoroacetic acid (TFA, Reagent Plus 99%) and sodium polyphosphate (crystals, +80 mesh, 96%) were supplied by Sigma-Aldrich (St. Louis, USA). Seventeen antioxidants were obtained from Sigma-Aldrich Pty. Ltd. (Castle Hill, NSW, Australia) that included: butylated hydroxyanisole, caffeic acid, (+)-catechin hydrate, 2,6-di-*tert*-butyl-4-hydroxymethylphenol, 2,6-di-*tert*-butyl-4-hydroxytoluene, ethoxyquin, ferulic acid, gallic acid, 4-hydroxycinnamic acid, lauryl gallate, nordihydroguaiaretic acid, octyl gallate, propyl gallate, quercetin, rosmarinic acid, *tert*-butylhydroquinone and vanillic acid. Potassium permanganate (AR Grade), hydrochloric acid (32% w/w, Analytical Reagent) and formaldehyde (37%) were obtained from Chem Supply (Gilman, SA, Australia). Sulphuric acid (98%) was supplied by Merck (Kilsyth, Vic., Australia). The permanganate reagent was prepared daily by dissolution of sodium polyphosphate in deionised water, adding potassium permanganate and adjusting to pH 2.5 using sulphuric acid.

2.2. Analyte preparation

All antioxidants were prepared separately as standard stock solutions at 1 mg mL^{-1} in 100% methanol and 100% acetonitrile. When injected in reversed phase mode the appropriate stock solution was diluted 1:2 with deionised water. In HILIC mode the acetonitrile stock was diluted with neat acetonitrile (thus the injection solvent was 100% acetonitrile).

The 2D-HPLC separation of a coffee was completed on an extraction of 5 g Ristretto brand Nespresso coffee (Nespresso, North Sydney, NSW, Australia) with 30 mL hot water by a Delonghi Nespresso Lattissima coffee machine (model number EN520W). The extract was filtered with a 0.45 μm syringe filter and made to a concentration of 95% acetonitrile prior to analysis.

Fresh urine samples were collected daily and preserved by adding a 7.5 M hydrochloric acid solution (50 μL per 1 mL of urine) and stored at 4 °C until required. Immediately prior to analysis, the preserved sample was diluted 10-fold with deionised water and filtered with a 0.45 μm syringe filter.

2.3. Instrumentation

Chromatographic analysis was performed with two Agilent 1260 chromatographs (Agilent Technologies, Mulgrave, Vic., Australia). The antioxidant investigation was completed on a system comprised of a binary capillary pump with solvent degasser; a 1290 Infinity binary pump with solvent degasser; an auto-sampler; a 1290 Infinity thermostatted column compartment with an in-built 8 port, 2 position switching valve and two DAD modules that monitored absorbance at 254 nm and 280 nm. The switching valve was configured according to Fig. 1 allowing analyses to be conducted on two separate columns concurrently with a gradient elution being performed on one column whilst the other column was being re-equilibrated. Chromatographic analysis of urine was completed with an Agilent Technologies 1260 Series liquid chromatography system, equipped with a quaternary pump (incorporating a vacuum degasser), column thermostat, diode array detector and autosampler (Agilent Technologies, Vic., Australia). Agilent Chemstation software was used for system control and data acquisition.

Chemiluminescence detection was employed by merging 2 M aqueous formaldehyde with the exit line from the column at a T-piece prior to entering a coiled-tubing detection flow cell [30]. The flow-cell was mounted flush against the window of a photomultiplier tube (Electron Tubes model 9828SB; ETP, NSW, Australia) encased in a light tight housing and powered by a stable power supply at 900 V. The potassium permanganate chemiluminescence reagent and formaldehyde solution were propelled at 1 mL min^{-1} using two Model 12-6 Dual Piston Pumps (Scientific Systems, PA,

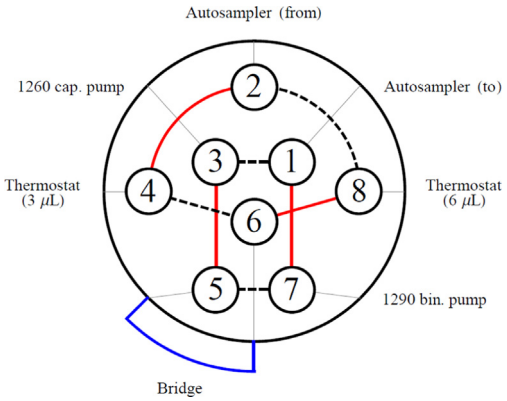

Fig. 1. Schematic representation of dual injection switching valve.

USA). A Hewlett-Packard 35900E analogue to digital converter was used to convert detector signals (Agilent Technologies).

The HPLC columns were thermally equilibrated for 1 h when the temperature was changed and trifluoroacetic acid was added to all mobile phases prior to analysis at a concentration of 0.1% v/v.

2.4. HPLC columns

Ten reverse phase columns were chosen to investigate optimisation *via* standard solution, these included: Poroshell 120 EC-C18 (100 mm × 4.6 mm, 2.7 μm, Agilent Technologies, Mulgrave, Vic., Australia); Poroshell 120 EC-CN (100 mm × 4.6 mm, 2.7 μm, Agilent); Luna NH_2 (100 mm × 4.6 mm, 5 μm, Phenomenex, Lane Cove, NSW Australia); Kinetex PFP (100 mm × 4.6 mm, 2.6 μm, Phenomenex); Onxy Monolithic C18 (100 mm × 4.6 mm, Phenomenex); a butyl phenyl cyano particle packed column and a propyl phenyl cyano particle packed column (both 100 mm × 4.6 mm, 5 μm); both packed in-house according to the packing procedure described in [31]; a cyano modified silica monolith, a propyl phenyl modified silica monolith and mixed mode modified silica monolith (all 100 mm × 4.6 mm, prepared according to the method described previously [32,33]).

For the optimisation using a complex sample as the standard mixture, the following 13 columns were used (pore diameter was 100 Å unless otherwise stated): Synergi Hydro-RP (250 mm × 4.6 mm, 4 μm, 80 Å pore diameter, Phenomenex); Zorbax Eclipse AAA (150 mm × 4.6 mm, 3.5 μm, Agilent); Zorbax Eclipse C18 XDB (150 mm × 4.6 mm, 5 μm, Agilent); Poroshell EC-C18 (100 mm × 4.6 mm, 2.7 μm, Agilent); Poroshell EC-CN (100 mm × 4.6 mm, 2.7 μm, Agilent), Poroshell EC-C18 (50 mm × 4.6 mm, 2.7 μm, Agilent); Poroshell EC-C8 (100 mm × 4.6 mm, 2.7 μm, Agilent); Synergi Polar-RP (150 mm × 2 mm, 4 μm, 80 Å pore diameter, Phenomenex), Aqua C18 (150 mm × 2.0 mm, 3 μm, 125 Å pore diameter, Phenomenex), Luna NH_2 (150 mm × 4.6 mm, 5 μm, Phenomenex), Aqua C18 (150 mm × 2.0 mm, 5 μm, 200 Å pore diameter, Phenomenex); Phenyl XDB Eclipse (150 mm × 4.6 mm, 3.5 μm, Agilent); and Chromolith Performance RP 18e (100 mm × 3 mm, Merck, Kilsyth, Vic., Australia).

2.5. HPLC simulation protocol

Retention parameters for matched HPLC peaks (2 gradient times at 2 temperatures) were entered into DryLab® (Molnar-Institute for applied chromatography, Berlin, Germany) for *in-silico* optimisation. To compare selectivity performance of the different HPLC columns, and remove retention time as a factor, the temperature and linear gradient times were optimised so that the last eluted compound had a retention of approximately 10 times the column void time (t_0). Following optimisation, each column was compared *via* Gilar's fractional surface coverage (bins) method [19,29] using a Mathematica 9.0 notebook (distributed by Hearn Scientific, South Yarra, Vic., Australia) developed in-house. According to Gilar et al. [19,29] the total number of bins is equal to the number of peaks, n_{peaks}; as this does not always divide evenly, the number of bins along each axis was calculated by taking the square root of n_{peaks} and rounding up to the nearest integer. The two columns that had the greatest orthogonality when paired were judged to be the optimal for 2D-HPLC. Instances where multiple combinations were deemed optimal were resolved by the analyst's best judgement.

2.6. One-dimensional HPLC methods

Single injections were made to fulfil the requirements of DryLab® for all antioxidant standards (individually) and the urine matrix were run on each of the respective columns using the following conditions: *Run* 1: 20 min gradient at 30 °C, *Run* 2: 60 min gradient at 30 °C, *Run* 3: 20 min gradient at 45 °C and *Run* 4: 60 min gradient at 45 °C. This was completed for the antioxidants with both aqueous solutions of both acetonitrile and methanol; the urine was separated using methanol only.

The mobile phase gradients had an initial composition of 5% organic solvent (acetonitrile or methanol) that increased to 100% over the allocated gradient time, except when operated in HILIC mode on the NH_2 column which had an initial mobile phase composition of 2% water in acetonitrile increasing to 30% over the allocated gradient time. All experiments were operated at 1 mL min^{-1}. Injections of antioxidants and urine had a volume of 5 μL and 20 μL, respectively, unless otherwise stated.

2.7. Two-dimensional HPLC methods

The concentration of the injection solvent relative to the initial mobile phase composition must be strictly controlled to ensure proper peak shape. However, in a 2D-HPLC separation when a gradient analysis is used in the first dimension that increases from a low to high percentage of organic modifier, the second dimension's injection solvent strength is continually increasing. To control the second dimension injection solvent composition, a counter gradient with a flow rate of 2 mL min^{-1} was combined with the first dimension eluent through a T-piece that was located prior to the fraction collector. The counter gradient was adjusted so that when mixed with the first dimension eluent a 20% acetonitrile solution was obtained. The conditions for the counter gradients were calculated according to previous work [34]. A minimum re-equilibration of 3 column volumes was flushed through the column prior to all second dimension analyses.

The 17 antioxidant standards were prepared as a mixture at 1 mg mL^{-1} in 100% acetonitrile and diluted 1:2 with acetonitrile. This mixture was separated in the comprehensive off-line mode of 2D-HPLC with the NH_2 column in the first dimension and the pentafluorophenyl (PFP) column in the second dimension. These columns were selected on the basis of predicted elution times with a maximum retention time of $10 \times t_0$ and a comparison with the bins method (see electronic Supplementary information for a full list of column comparisons). The 2D-HPLC separation parameters were optimised *in-silico* using the same preliminary screening data used to compare column selectivity. The first dimension was operated in HILIC mode whereby the initial mobile phase composition was 2% water in acetonitrile that increased to 9% over 20 min. Analysis was carried out at 30 °C with a 10 μL injection, employing flow rate of 0.5 mL min^{-1}. The second dimension was operated at 45 °C with an acetonitrile mobile phase using the gradient conditions in Table 1. Fractions of the first dimension eluent were collected every 50 μL with a Gilson FC204 fraction collector (John Morris Scientific, Balwyn, Vic., Australia, with the counter gradient

Table 1
Gradient elution profile for the second dimension, separations were completed at 2.5 mL min^{-1}.

Time (min)	Water (%)	Acetonitrile (%)
0	95	5
0.3	78.5	21.5
0.5	78.5	21.5
0.9	67	33
1.15	61	39
1.6	55	45
1.9	55	45
2.15	0	100
2.25	0	100
2.26	95	5
3.0	95	5

the total collected volume was 250 μL) and 100 μL was injected onto the second dimension.

The separation of the coffee extract was completed in the comprehensive off-line mode of 2D-HPLC using the same columns as the separation of the antioxidant standards with the following modifications: 100 μL was injected into an initial mobile phase composition in the first dimension of 2% water in acetonitrile that increased to 4% over 4 min, with a final increase to 25% water over a further 2 min. The second dimension was operated in the same conditions as previously stated; except that the %B was halved at each step of the gradient profile outlined in Table 1. Fractions were collected every 50 μL, which corresponded to 4 fractions per peak measured at 4σ width, and considering the volume contributed by the counter gradient a total of 250 μL was collected; 100 μL was injected onto the second dimension.

The 2D-HPLC separation of urine was also performed in the comprehensive off-line mode with the Synergi Polar-RP column in the first dimension and Poroshell Cyano column in the second. A 100 μL aliquot was injected on the first dimension that separated *via* a linear gradient that began with an initial concentration of 5% methanol and increased to 100% methanol over 20 min at a flow rate of 0.5 mL min^{-1} and column temperature of 20 °C. Fractions were collected at 0.09 min intervals that corresponded to 3 fractions/peak – calculated from 4σ peak width – and merged with the counter gradient prior to the fraction collector that adjusted the second dimension injection solvent to 10% methanol. 100 μL of each fraction was then injected onto the second dimension and separated with a 10 min linear mobile phase gradient from 10% methanol to 100% methanol, using a flow rate of 1.5 mL min^{-1} and a column temperature of 60 °C.

3. Results and discussion

3.1. Standards

Even though the HPLC simulation software can match peaks from recorded chromatograms by comparing peak areas, in this work it was found that the program needed assistance to distinguish between overlapping peaks and has trouble with changes of elution order. Therefore, each of the antioxidants from the list of standards was injected individually 4 times (with t_G of 20 and 60 min each at 30 °C and 45 °C). These 4 injections were completed on 10 HPLC columns with different selectivity and compared using the bins method [19,29] to find the combinations with the greatest separation divergence. Seventeen antioxidants were included as standards. It has been shown that this is an appropriate number to estimate the 2D-HPLC peak capacity that can be extrapolated to a larger number of components [19]. This is probably an accurate method to compare retention behaviour of peaks with different experimental parameters and on different HPLC columns; however, this was quite time consuming as an estimate of 1292 injections needed to be performed. Although, the 2D-HPLC instrument was configured so that the autosampler could be shared between the 2 pumps and detectors, thus saving significant re-equilibration time, see Fig. 1.

After the injections had been completed the data for each peak was entered into the simulation software. Each of the separations were designed so that the last eluted peak would have a retention time of approximately $10 \times t_0$ and the temperature was adjusted to provide the largest minimum peak resolution between adjacent peaks. These optimised retention times were copied into a Microsoft Excel spreadsheet and compared *via* the fractional surface coverage (bins) method with Wolfram Mathematica 9 and algorithms written in-house. It was found that the combinations: NH_2 (HILIC) × PFP (acetonitrile), NH_2 (HILIC) × CN monolith (acetonitrile) and NH_2 (HILIC) × C18 (acetonitrile) all had the maximum orthogonality of 57%. The NH_2 (HILIC) × PFP combination was selected to compare the performance of the HPLC simulation software when applied to two-dimensional chromatography and to test if the same orthogonality was achieved when scaling up the complexity of a separation.

Prior to two-dimensional analysis, the operating conditions were adjusted with DryLab® so that the elution times in both dimensions were appropriate for 2D-HPLC. The resulting 2D-HPLC separation was completed in the off-line mode, whereby fractions were collected in vials, loaded into the autosampler and sequentially re-injected. The results of the combination of these two columns is illustrated in Fig. 2. The retention predictions for both the NH_2 and PFP columns closely matched those of the simulation software (indicated by circles). However, the later eluting peaks in the first dimension, NH_2 (HILIC), were more strongly retained than what was predicted.

A counter gradient was essential to control the second dimension injection solvent composition (20% acetonitrile in water) by combining it with the eluent stream from the first dimension generated with a second HPLC pump *via* a T-piece located immediately prior to the fraction collector, according to Stevenson et al. [34]. It was expected that the orthogonality would be slightly lower than was predicted after the introduction of the counter gradient as the initial second dimension mobile phase composition was increased to 20% acetonitrile; the ideal separation began at 5%. After the addition of the counter gradient the predicted orthogonality was 38%, but this was 44% for the actual 2D-HPLC separation of the antioxidant mixture. This can be attributed to the retention time of a single eluted peak that was slightly less than predicted (ca. 0.01

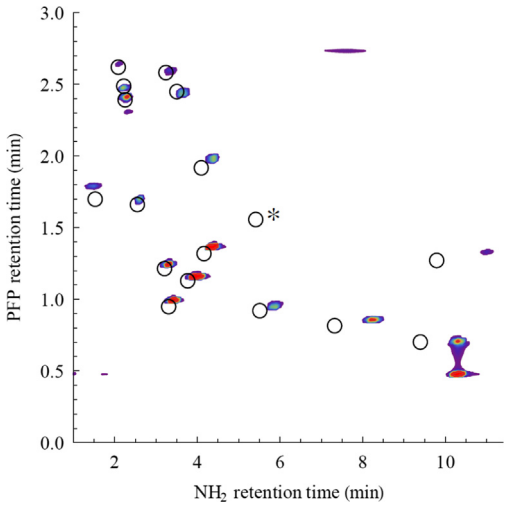

Fig. 2. 2D-HPLC plot of antioxidant mixture with NH_2 column in the first dimension and PFP in the second. The circles represent the predicted retention times as generated by the HPLC simulation software. The star (*) is placed at the retention co-ordinates of a negative peak (positive when recorded at 254 nm).

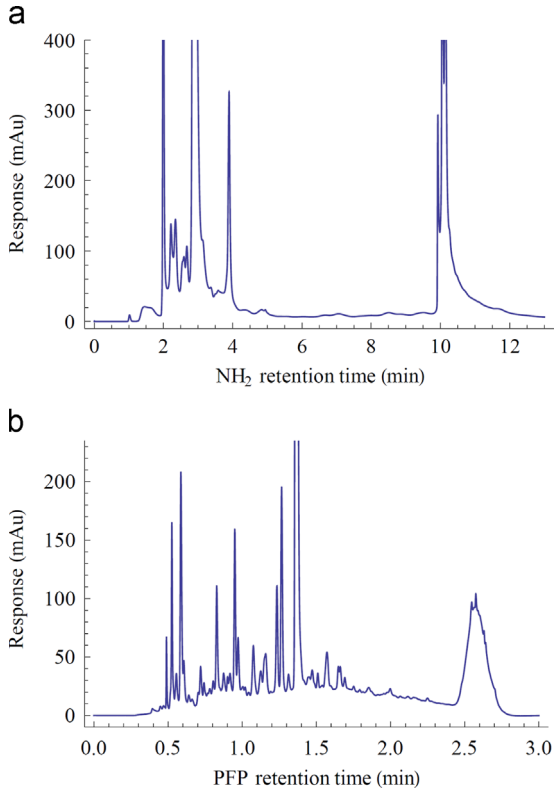

Fig. 3. One-dimensional HPLC separations of a coffee extract on the (a) NH_2 and (b) PFP stationary phases with aqueous acetonitrile.

scaled units), but was enough for it to occupy an extra bin. It was expected that these parameters would be maintained when the separation was scaled up to a complex matrix. The separation spaces were divided into 25 even bins for the calculation of *O*, thus the maximal achievable orthogonality was 68% ($100 \times 17/25$).

An extract of coffee was used to represent a complex sample matrix as the antioxidant content of this has previously been reported by Mnatsakanyan and co-workers [35,36]. Consequently, it was considered that a series of antioxidant standards would be suitable to optimise this extraction. Each of the one-dimensional analyses are illustrated in Fig. 3. In the NH_2 dimension most of the components eluted in a narrow window between 2 and 4 min; however, the total analysis time need to be extended beyond 12 min to ensure all peaks were accounted for. Conversely, the PFP dimension eluted all chemical species evenly during the course of the 3 min analysis. When these separations were coupled, the 2D-HPLC chromatogram illustrated by Fig. 4 was achieved. The bi-modal distribution that was observed in Fig. 3(a) is again present in the 2D-HPLC plot. However, even though a large proportion of the separation space was not used in the middle of the first dimension, the measured orthogonality of the two-dimensional separation (O=39%, 2D peak retention times calculated using our previously published method [37]) was equal to that predicted from the simulated chromatograms; the separation space was divided into 169 bins to calculate *O*. When only considering the region between 2 and 4 min in the first dimension, bound by the white box in Fig. 4, the majority of the separation space was occupied (O=64%). In this instance, optimisation was successfully achieved through utilisation of a standard mixture to represent a more complex matrix. However, the bi-modal separation in the first dimension highlighted the main problem with analysis of unknown complex mixtures with 2D-HPLC: the resulting separation of the unknown mixture still provided large regions of empty space, even though select regions displayed an impressive orthogonality. Moreover, the total analysis time to optimise this separation of the 17 antioxidants on the range of columns

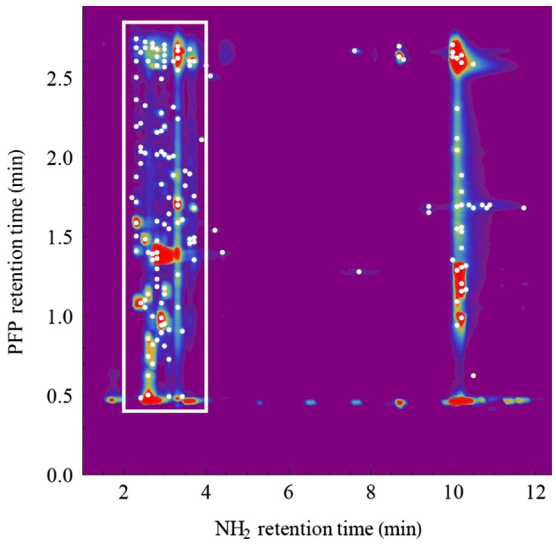

Fig. 4. Two-dimensional separation of a coffee extract in the off-line mode with the NH_2 column in the first dimension and PFP column in the second. White dots represent peak-maxima as detected *via* peak picking algorithms.

was estimated to be 168 h, this is excessive and suspected to not be practical for many separation problems.

3.2. Optimising with an unknown sample matrix

The alternative way of optimising the separation dimensions for 2D-HPLC is to use the sample itself as the standard mixture; indeed, this is the only method possible when the contents of the sample are completely unknown. This was aided by gaining as much information about the sample as possible from multiple detection strategies. In this case both of UV absorbance and chemiluminescence detection were used to optimise the 2D-HPLC separation of human urine. Potassium permanganate chemiluminescence is a selective detection method that has been previously used [30,38] to rapidly screen for neurotransmitter metabolites. *In lieu* of identified standards the retention times of compounds within the sample had to be matched between separations with different operating conditions, including gradient times, temperatures and different HPLC columns. The retention times of peaks on the different HPLC columns were matched by comparing the relative ratios of peak areas between the two detection strategies. By comparing the ratio between these two detection techniques the analyst can have more confidence that the peaks that are being compared between columns are in-fact from the same compounds. This is made possible because these strategies are selective to different attributes of the sample constituents, a chromophore in the case of UV absorbance detection, and chemiluminescence activity with acidic potassium permanganate. A representative separation of urine on a cyano column with UV absorbance and chemiluminescence is illustrated in Fig. 5.

Comparing the relative retention times of compounds *via* different separation environments by comparing the relative peak heights of different detection protocols proved to be a difficult and time consuming task. Although there can be no exact comparison between UV absorbance and chemiluminescence intensities. Seven compounds were matched over the recorded chromatograms by manually comparing the relative heights of peaks between the two detection strategies of the different columns. According to Gilar et al. [19] at least 25 data points are required to accurately extrapolate the final orthogonality of a 2D-HPLC separation with the bins method (this was the starting point of their comparisons). The number of peaks that were detected and matched between chromatograms was much less than this, but each column combination was still compared *via* the

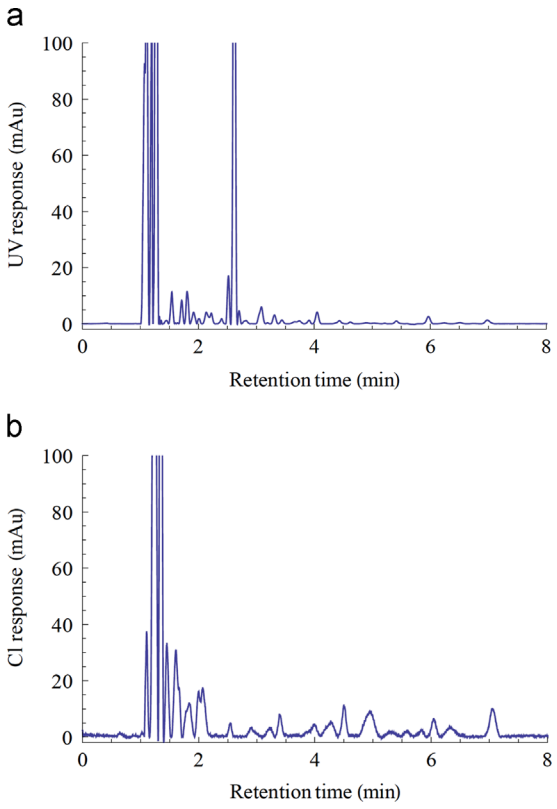

Fig. 5. Separation of urine on a cyano column with UV absorbance and permanganate chemiluminescence. The relative peak heights were compared between chromatograms to identify likely candidates for peak matching. Baselines were corrected with an asymmetric least squares approach ($\lambda=1$, $p=1.0\times10^{-7}$) [39] (note: the chemiluminescence was installed downstream from the UV absorbance detector thus the retention times of peaks are slightly delayed).

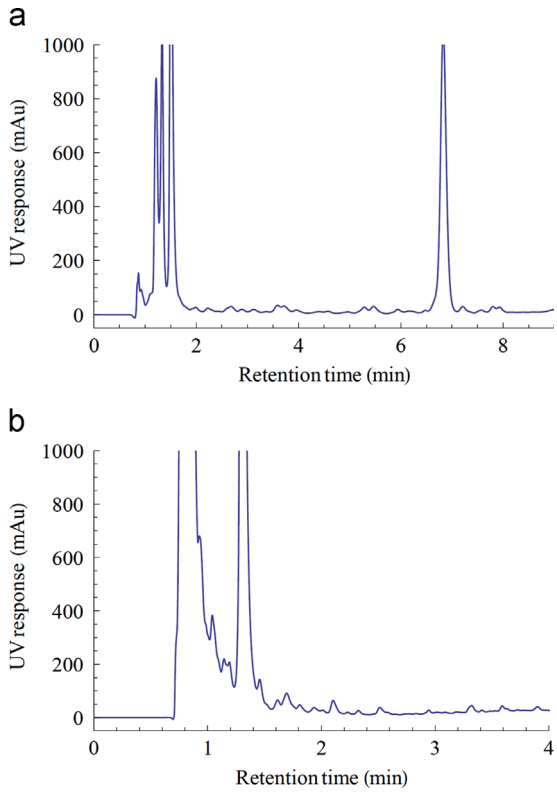

Fig. 6. One-dimensional HPLC separations of urine on the (a) Synergi Polar-RP and (b) cyano stationary phases with aqueous methanol.

bins approach. The same optimisation protocol as in the previous section was used to create the simulated chromatograms with DryLab®. Both the combinations of Synergi-Hydro × C18, and the Synergi-Polar RP × CN were found to have the best orthogonalities of 35%; calculated from 9 bins (3 × 3). However, because there were only 7 peaks available for comparison the size of each bin is much larger than when more peaks are present and the likelihood of these bins being occupied are greater; the maximum orthogonality was 78% (100 × 7/9). Nevertheless, after visual inspection it was decided that the combination of Synergi-Polar RP × CN would be used as the optimal column combination and were chosen for the two-dimensional HPLC separation of urine.

The experimental parameters for each dimension were reconfigured so that 2D-HPLC analysis did not take an excessive amount of time. The first and second dimension separations are illustrated in Fig. 6. Fractions of the first dimension were collected every 45 μL (0.09 min, total collected fraction including counter gradient was 245 μL) from the first dimension and re-injected into the second dimension, which took 4 min to complete. The resultant 2D-HPLC plot is presented in Fig. 7. The retention times of 56 2D-HPLC peaks were determined from the raw data with peak picking algorithms [37] and the orthogonality of the separation was calculated according to the bins method to be 30%. This is in close agreement with the orthogonality that was predicted, which is somewhat surprising as only 7 peaks were matched between chromatograms; the orthogonality was 35%. It is likely that if more peaks could be matched between chromatograms, then the final orthogonality would be even closer to that predicted. The compounds that were matched successfully here were ultimately a good representation of the whole sample matrix; it is unrealistic that this will happen every time the optimisation protocol is employed. Optimisation of the entire sample matrix will only be achieved when more information about the separation is collected, either by using extra detection strategies in parallel, such as time of flight mass spectrometry and sifting through the slew of mass information. This will be investigated further in future work.

This paper looked at two protocols for optimising the 2D-HPLC dimensions on the basis of selectivity. Only the stationary and mobile phases were investigated here even though a more extensive study of experimental parameters will result in a better overall separation. However, expanding the mobile phase selection and investigating effects of temperature and pH further amplifies the problems associated with dimension selection by greatly increasing both the number of screening runs required to predict the outcome and the possible combinations. Both methods of optimising the combination of columns for 2D-HPLC by comparing the orthogonality from simulated 2D-HPLC separations proved successful at approximating the final outcome. However, the use of a series of standards to describe the complex sample matrix had an orthogonality closer to the final outcome ($O_{prediction}=38\%$; $O_{actual}=39\%$), but, it is impossible to use standards in cases where the analyst has little to no knowledge of the contents of their sample, which is often the case in natural product chemistry and other complex separations. The optimal column selection when using a complex sample matrix as the optimisation mixture had an $O_{predicted}$ of 35% and an O_{actual} of 30%. This was a close approximation; however, the successful match of more peaks was required to increase the prediction's accuracy. Using the complex matrix itself required far fewer injections to satisfy the HPLC simulator's requirements than when using standards, but this required a significant amount of time by the analyst to sift through the relative peak heights from the 2 detection strategies for only 7 matches. Conversely, using standards – as long as they are appropriate to the sample – allowed for more confidence in the simulation, but required a significant amount of instrument time and HPLC solvent. In conclusion, both of these optimisation strategies will accurately predict the orthogonality of the final separation but

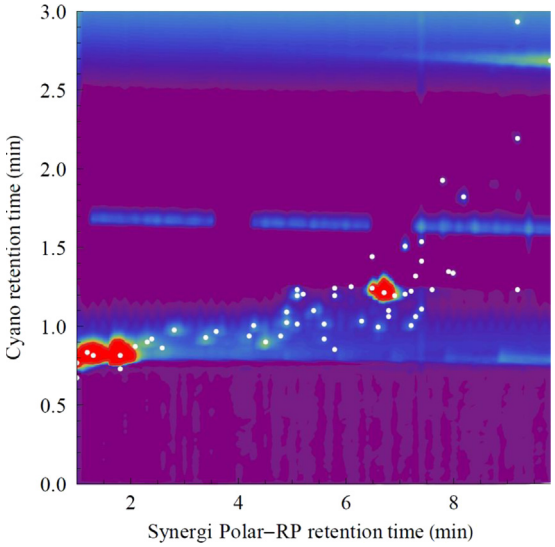

Fig. 7. Two-dimensional HPLC separation of urine with a Synergi Polar-RP column in the first dimension and cyano in the second recorded with an UV detector at 230 nm. White dots represent peak-maxima as detected *via* peak picking algorithms.

required a significant amount of time to complete and might only be useful designing a 2D-HPLC for repeat analysis.

Acknowledgements

This work was partially funded by an Australian Research Council Discovery Grant (DP140100439). The authors would like to acknowledge the receipt of a Deakin University postgraduate research award (DNB), an Australian Postgraduate Research award (BJH), and an Alfred Deakin postdoctoral research fellowship (PGS).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.11.037.

References

[1] R.J. Vervoort, A.J. Debets, H. Claessens, C. Cramers, G. de Jong, J. Chromatogr. A 897 (2000) 1–22.
[2] J.C. Giddings, Anal. Chem. 56 (1984) 1258A–1270A.
[3] P.J. Slonecker, X. Li, T.H. Ridgway, J.G. Dorsey, Anal. Chem. 68 (1996) 682–689.
[4] E. Grushka, N. Grinberg, Advances in Chromatography, CRC Press, Boca Raton, FL, 2012.
[5] L.R. Snyder, P.W. Carr, S.C. Rutan, J. Chromatogr. A 656 (1993) 537–547.
[6] J.W. Dolan, J. Chromatogr. A 965 (2002) 195–205.
[7] P.J. Schoenmakers, S. van Molle, C.M.G. Hayes, L.G.M. Uunk, Anal. Chim. Acta 250 (1991) 1–19.
[8] N.E. Hoffman, S.-L. Pan, A.M. Rustum, J. Chromatogr. A 465 (1989) 189–200.
[9] J. Layne, T. Farcas, I. Rustamov, F. Ahmed, J. Chromatogr. A 913 (2001) 233–242.
[10] W. Naidong, Y.-L. Chen, W. Shou, X. Jiang, J. Pharm. Biomed. Anal. 26 (2001) 753–767.
[11] R.A. Shalliker, G. Guiochon, J. Chromatogr. A 1216 (2009) 787–793.
[12] R.A. Shalliker, G. Guiochon, Analyst 135 (2010) 222–229.
[13] Z. Liu, D.G. Patterson, M.L. Lee, Anal. Chem. 67 (1995) 3840–3845.
[14] N. Tanaka, T. Tanigawa, K. Kimata, K. Hosoya, T. Arai, J. Chromatogr. 549 (1991) 29–41.
[15] L.R. Snyder, J.W. Dolan, P.W. Carr, J. Chromatogr. A 1060 (2004) 77–116.
[16] Y. Zhang, P.W. Carr, J. Chromatogr. A 1216 (2009) 6685–6694.
[17] A.R. Johnson, C.M. Johnson, D.R. Stoll, M.F. Vitha, J. Chromatogr. A 1249 (2012) 62–82.
[18] E. Cruz, M.R. Euerby, C.M. Johnson, C.A. Hackett, Chromatographia 44 (1997) 151–161.
[19] M. Gilar, J. Fridrich, M.R. Schure, A. Jaworski, Anal. Chem. 84 (2012) 8722–8732.
[20] S.E. Reichenbach, X. Tian, Q. Tao, D.R. Stoll, P.W. Carr, J. Sep. Sci. 33 (2010) 1365–1374.
[21] M. Mnatsakanyan, P.G. Stevenson, D. Shock, X.A. Conlan, T.A. Goodie, K.N. Spence, et al., Talanta 82 (2010) 1349–1357.
[22] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, J. Sep. Sci. 28 (2005) 1694–1703.
[23] J.W. Dolan, L.R. Snyder, M.A. Quarry, Chromatographia 24 (1987) 261–276.
[24] A. Soliven, I.A. Haidar Ahmad, M.R. Filgueira, P.W. Carr, J. Chromatogr. A 1273 (2013) 57–65.
[25] X. Wang, D.R. Stoll, A.P. Schellinger, P.W. Carr, Anal. Chem. 78 (2006) 3406–3416.
[26] K. Horie, H. Kimura, T. Ikegami, A. Iwatsuka, N. Saad, O. Fiehn, et al., Anal. Chem. 79 (2007) 3764–3770.
[27] A.H. Schmidt, I. Molnár, J. Pharm. Biomed. Anal. 78–79 (2013) 65–74.
[28] L.R. Snyder, J. Chromatogr. A 92 (1974) 223–230.
[29] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, Anal. Chem. 77 (2005) 6426–6434.
[30] J.L. Adcock, P.S. Francis, N.W. Barnett, Anal. Chim. Acta 601 (2007) 36–67.
[31] V. Wong, R.A. Shalliker, G. Guiochon, Anal. Chem. 76 (2004) 2601–2608.
[32] A. Soliven, G.R. Dennis, G. Guiochon, E.F. Hilder, P.R. Haddad, R.A. Shalliker J. Chromatogr. A 1217 (2010) 6085–6091.
[33] A. Soliven, G. Dennis, E. Hilder, R. Andrew Shalliker, P. Stevenson, Chromatographia 77 (2014) 663–671.
[34] P.G. Stevenson, D.N. Bassanese, X.A. Conlan, N.W. Barnett, J. Chromatogr. A 1337 (2014) 147–154.
[35] M. Mnatsakanyan, T.A. Goodie, X.A. Conlan, P.S. Francis, G.P. McDermott, N.W. Barnett, et al., Talanta 81 (2010) 837–842.
[36] M. Mnatsakanyan, P.G. Stevenson, X.A. Conlan, P.S. Francis, T.A. Goodie, G.P. McDermott, et al., Talanta 82 (2010) 1358–1363.
[37] P.G. Stevenson, M. Mnatsakanyan, G. Guiochon, R.A. Shalliker, Analyst 135 (2010) 1541–1550.
[38] B. Holland, X. Conlan, P. Stevenson, S. Tye, A. Reker, N. Barnett, et al., Anal. Bioanal. Chem. (2013) 1–8.
[39] P.G. Stevenson, X.A. Conlan, N.W. Barnett, J. Chromatogr. A 1284 (2013) 107–111.