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The assessment of π–π selective stationary phases for two-dimensional HPLC analysis of foods: Application to the analysis of coffee

Mariam Mnatsakanyan[a], Paul G. Stevenson[a,c], David Shock[a], Xavier A. Conlan[d], Tiffany A. Goodie[e], Kylie N. Spencer[e], Neil W. Barnett[e], Paul S. Francis[d,e], R. Andrew Shalliker[a,b,*]

[a] Australian Centre for Research on Separation Science (ACROSS), University of Western Sydney Node, School of Natural Sciences, Parramatta, NSW, Australia
[b] Nanoscale Organisation and Dynamics Group, University of Western Sydney, Parramatta, NSW, Australia
[c] Centre for Complementary Medicine Research, University of Western Sydney, Parramatta, NSW, Australia
[d] Institute for Technology Research and Innovation, Deakin University, Geelong, Victoria 3217, Australia
[e] School of Life and Environmental Sciences, Deakin University, Geelong, Victoria 3217, Australia

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ABSTRACT

Differences between alkyl, dipole–dipole, hydrogen bonding, and π–π selective surfaces represented by non-resonance and resonance π-stationary phases have been assessed for the separation of 'Ristretto' café espresso by employing 2DHPLC techniques with C18 phase selectivity detection. Geometric approach to factor analysis (GAFA) was used to measure the detected peaks (N), spreading angle (β), *correlation*, practical peak capacity (n_p) and percentage usage of the separations space, as an assessment of selectivity differences between regional quadrants of the two-dimensional separation plane. Although all tested systems were correlated to some degree to the C18 dimension, regional measurement of separation divergence revealed that performance of specific systems was better for certain sample components. The results illustrate that because of the complexity of the 'real' sample obtaining a truly orthogonal two-dimensional system for complex samples of natural origin may be practically impossible.

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1. Introduction

Due to the complexity of natural products, designing methods for analysis and characterization can be a formidable task. Natural products are known for the range and novelty of their chemical diversity, often containing thousands of components, many with physical and chemical similarities, often present as low abundant species. It is because of this chemical complexity that two-dimensional high performance liquid chromatography (2DHPLC) has been attracting more interest in the field of natural product chemistry. In some cases 2DHPLC has shown potential for obtaining good separation of highly complex samples [1], including natural products [1–5], although, many two-dimensional separations employed in the field have not undergone a rigorous test of orthogonality, and few report the practical peak capacity of the system.

The principal advantage of two-dimensional separation is that it provides, relative to one dimensional, a greatly enhanced peak capacity [6], provided each of the dimensions offer divergent retention behaviour. In principle, the maximal theoretical peak capacity in 2DHPLC that employs orthogonal dimensions is equal to the product of the peak capacities of each respective one-dimensional separation, but the overall peak capacity reduces as a function of correlation between the systems [7–9]. In order to fully utilise the power of a two-dimensional separation, design of the system should be undertaken with due consideration to the nature of the sample [6]. That is, each of the dimensions within the separation system should ideally be selective towards a specific sample attribute. This ensures ordered displacement of sample components across the 2D space. In practice, a system comprising 'n' dimensions for 'n' sample attributes is impossible since multi-dimensional separations are largely limited to two-dimensions. Therefore, fully non-correlated selectivity for each dimension in a two-dimensional system is rarely found [10], especially for complex samples of natural origin. Thus it is important that in order to maximise system peak capacity, the operating conditions in the two-dimensional system should be carefully measured and subsequently optimised. Such studies require firstly a degree of understanding with respect to the behaviour of the solutes in each dimension. That is, selectivity studies must be undertaken with respect to both the stationary phase and also the mobile phase. These studies must be undertaken practically, since it is largely impossible to predict selectivity behaviour. Furthermore, due care

* Corresponding author at: Australian Centre for Research on Separation Science (ACROSS), University of Western Sydney Node, School of Natural Sciences, Victoria Road, North Parramatta, Parramatta, NSW 2150, Australia. Tel.: +61 2 9685 9951; fax: +61 2 9685 9915.
E-mail address: r.shalliker@uws.edu.au (R.A. Shalliker).

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must be paid to the types of compounds used to determine the 'selectivity' of the phase, as it must match that of the sample for an accurate measure of performance [11,12]. Thus, the sample itself should be used to test the selectivity changes, and herein lies the problem. Samples of natural origin are largely too complex for selectivity testing to be undertaken in a unidimensional sense, unless hyphenated methods of detection are employed that can track specific sample components as a function of the selectivity change. In complex samples, this tracking process is tedious, and to be effective, a large number of representative sample components must be tested. If the number is too small, or their distribution does not reflect that of the actual sample separation, then their reliability as a measure of selectivity performance may be questionable [11].

The most reliable approach to measuring selectivity differences is to employ the sample itself, as then selectivity changes are truly reflected in the separation of the natural product. However, for complex samples utilisation of the natural product itself during the design phase of separation is far from straight forward, as there are usually multitudes of components that co-elute, and changes in selectivity are likely to go unnoticed as one complex chromatogram looks very similar to another. Carr and co-workers [13], however, introduced the concept that a 2DHPLC system could in fact be utilised as a separation process as the first dimension, and then the second dimension serve as a selectivity detector. In that way, changes that are made to the first dimension can be assessed in the retention distribution in the second dimension. The relative change in selectivity of the different first dimensions can therefore be gauged. Selectivity in RP chromatography has been extensively studied. Cyano and phenyl phases showed little selectivity advantage over C18 columns in RP separations when initially investigated [14]. Further studies proved that there is an alternative selectivity for cyano and phenyl phases and theories on the interaction mechanisms have been put forth [15–17]. More recent studies have investigated the different selectivity observed between a phenyl phase using π–π interaction and a cyano phase using non-π-resonance or a dipole–dipole interaction [18]. Even the configuration of the phenyl phase induces changes in selectivity [19]. Fluoro-substituted columns have also shown alternative selectivity to alkyl and phenyl phases [20]. In the majority of these selectivity studies, a finite number of test analytes are used to characterize the selectivity. The current study will investigate selectivity with respect to the behaviour of a complex sample derived from natural origin containing a multitude of components. In this study the focus is on general selectivity differences rather than specific functional differences. To illustrate this process we assessed the separation of 'Ristretto' espresso on a number of π-selective stationary phases, employing 2DHPLC techniques with selectivity detection, with the view of maximising the separation power for extended studies on the analysis of coffee.

2. Experimental

2.1. Chemicals and samples

All solvents were of HPLC grade. Acetonitrile, methanol, tetrahydrofuran were from Lomb Scientific (Tarren Point, NSW, Australia). Milli-Q water (18.2 MΩ) was obtained in-house and used through all the experiments. Espresso 'Ristretto' coffee was obtained from the local market (Nespresso Australia, North Sydney, NSW, Australia). The coffee brews were home made using an 'espresso' coffee-making machine (Nespresso DēLonghi, Nestle Nespresso, SA, Australia). Coffee brews for analysis were prepared using a 30 mL shot and were diluted 1/4 in water prior to analysis. All samples prior to injection into the LC system were filtered through 0.45-μm pore filter.

2.2. Chromatography columns

All chromatography columns were supplied by Phenomenex (Lane Cove, NSW, Australia). Five different functionalities were tested: Luna 100 Å Cyano (CN), SphereClone ODS, Luna Phenylhexyl (PH), Synergi Hydro-RP 80 Å (C18 with polar end-capping) and a Luna pentafluorophenyl (PFP). All column formats were 150 mm × 4.6 mm, packed with 5 μm particles.

2.3. Chromatographic instrumentation

All chromatographic experiments were conducted using a Waters 600E Multi Solvent Delivery LC System equipped with Waters 717 plus auto injector, Waters 600E pumps, Waters 2487 series UV/VIS detectors and Waters 600E system controller. The chromatographic interface between the first and second dimensions consisted of two electronically controlled, two-position six-port switching valves fitted with micro-electric valve actuators.

2.4. Chromatographic separation

2.4.1. First-dimensional separations

First-dimensional separations were performed on either of the CN, C18 with polar end-capping, PH or PFP columns. Selectivity studies were undertaken in aqueous solvents of methanol, acetonitrile and THF, however, in this work we report only the results derived from the aqueous-methanol system as it yielded the greatest separation performance. Since the second dimension was to serve as the 'detector' (assessing the changes that result from the first dimension), the same mobile phase conditions that were employed in the first dimension were also used in the second dimension. All separations, in both dimensions, were operated under linear gradient conditions, starting with 100% aqueous mobile phase and finishing with 100% methanol mobile phase, at a gradient rate of 10% per min. All flow rates were 1 mL/min and injection volumes were 100 μL into the first dimension. Mobile phases were unbuffered for all experiments, despite the fact that coffee is known to contain a high number of carboxylic acids. Initial experiments were undertaken using acetate buffered mobile phases; however, the separation performance was essentially the same, perhaps even reduced (results not shown). Non-buffered mobile phases enhanced our ability to undertake mass spectral analysis in the negative ion mode and also reduced one further aspect of solvent mismatch between the respective first and second dimensions: That of pH shock in the second dimension.

2.4.2. Second-dimensional separations

The second dimension was conducted on the SphereClone C18 column, using gradient elution with an initial mobile phase of 100% water, running to a final mobile phase of 100% methanol, at a gradient rate of 10% min. The flow rate was 1 mL/min. The transfer volume from the first dimension to the second dimension was 200 μL. UV absorbance detection was set at 280 nm.

2.4.3. Operation

A 'comprehensive' or more precisely an 'incremental heart cutting' approach was used to express the two-dimensional peak displacement, by which a 200 μL heart-cut section was transferred to the second dimension, with subsequent second dimension

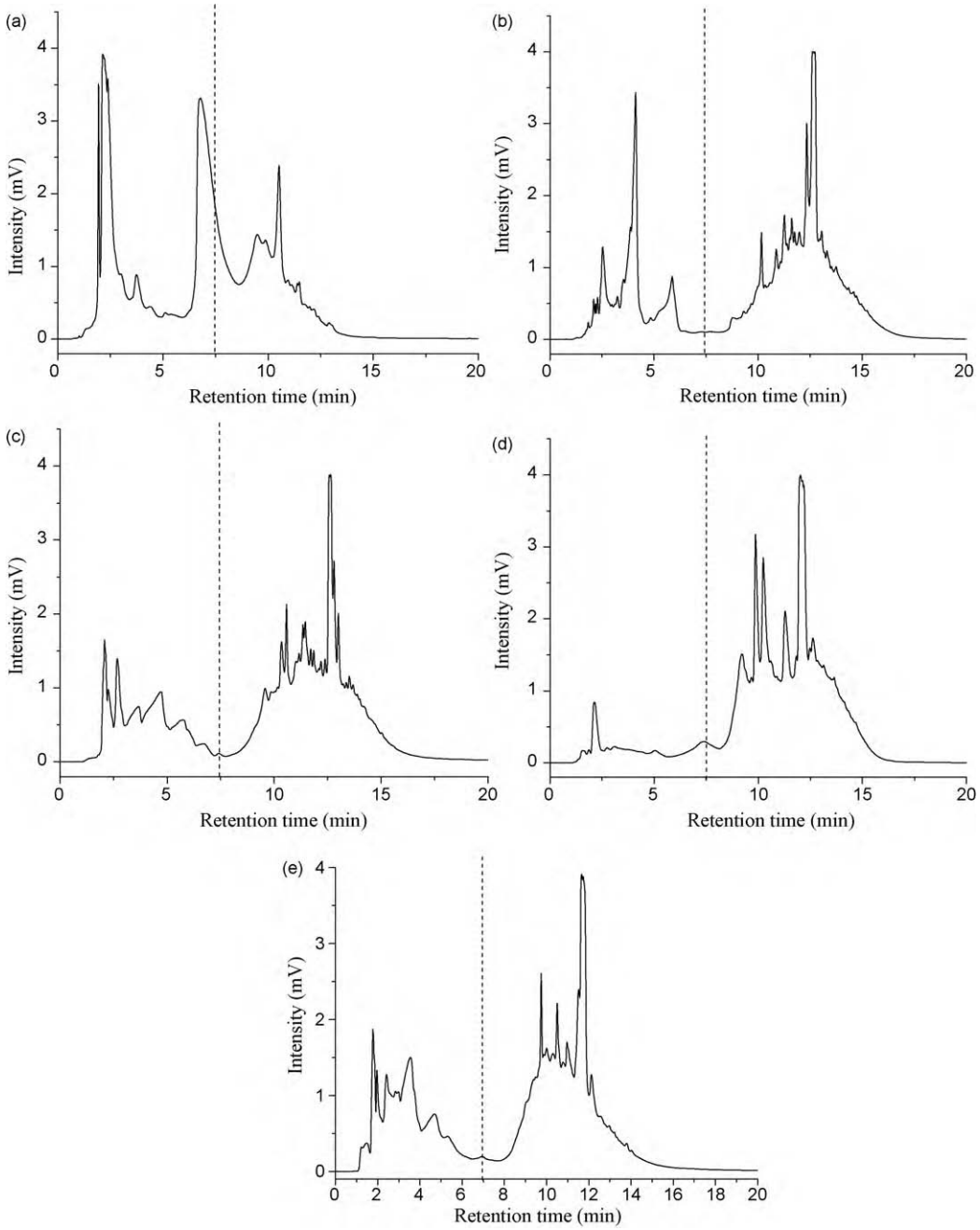

Fig. 1. One-dimensional separations of Ristretto café espresso on (a) Cyano, (b) Phenyl-hexyl, (c) Pentafluoro-phenyl, (d) Synergi Hydro C18 and (e) C18 phases. Mobile phase was aqueous/methanol, going from 100% water to 100% methanol. All conditions were identical for all phase systems.

separation being undertaken. The first-dimensional separation was repeated, following which another 200 μL first dimension fraction was transferred to the second dimension. This was repeated at every 0.4 mL across the entire first dimension separation, i.e. the first dimension separation was repeated a total of 34 times over a 20-h period.

2.5. Mass spectra analysis

A 6210 MSDTOF mass spectrometer (Agilent Technologies) was used with the following conditions: drying gas, nitrogen (7 mL min^{-1}, 350 °C); nebulizer gas, nitrogen (16 psi); capillary voltage, 4.0 kV; vaporiser temperature, 350 °C, and cone voltage, 60 V. All mass spectra data were handled using MassHunter Qualitative Analysis software (Agilent Technologies).

2.6. Data processing

Data plotting and calculation of retention information, including the statistical measures of the geometrical approach to factor analysis was performed using an in-house written program [22] using *Mathematica 7*.

The analysis of data, with respect to the measure of separation selectivity (i.e. geometric approach to factor analysis), as in this study, has been based solely on the displacement of UV absorbing species at 280 nm. Prior works have shown that the measure of separation 'orthogonality' in 2DHPLC is highly dependent upon the sample matrix, even if the selected species employed are contained within the real sample [11]. By restricting the analysis to UV absorbing species, the measure of selectivity was simplified since only UV detection was required. However, over the course of the

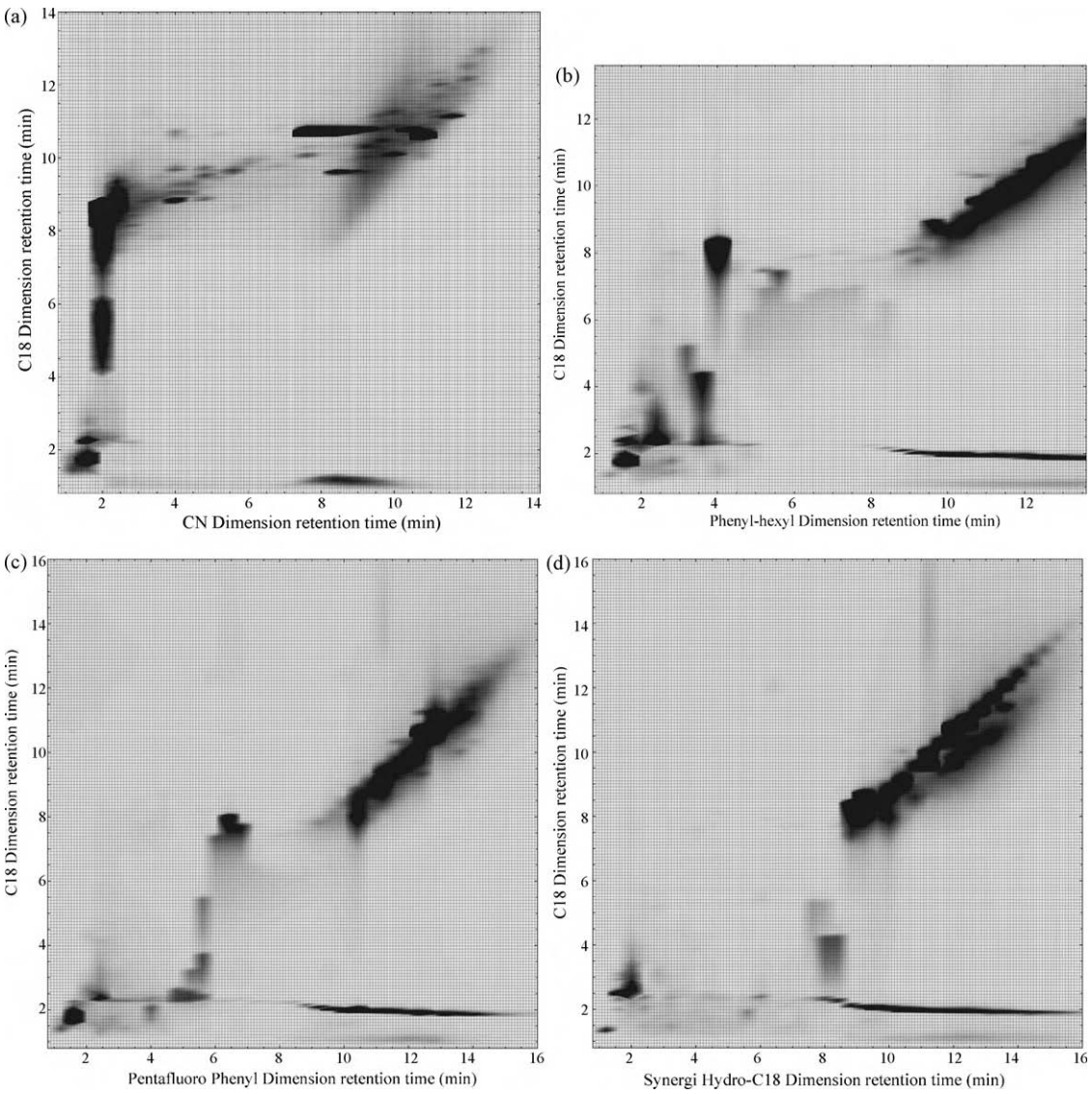

Fig. 2. Two-dimensional separations of Ristretto café espresso. First dimension (a) cyano, (b) phenyl-hexyl, (c) pentafluoro-phenyl and (d) Synergi Hydro-C18 and second dimension C18 phases. In both dimensions mobile phase was aqueous/methanol, going from 100% water to 100% methanol. All conditions identical for each phase system.

study we undertook the identification of some components using MS/MS detection. Some of these had limited UV, or even no UV response at 280 nm. It should therefore be noted, that these compounds, perhaps not included in the 'orthogonality' aspect of the study if included would likely alter some outcomes of the selectivity discussion.

3. Results and discussion

Manufacturers of chromatography columns are continually increasing the spectrum of selectivity that is available for the separation scientist. The selection of the most appropriate combination of phase systems for 2DHPLC can be daunting. In this work we simplified the array of stationary phases to focus on π–π selective surfaces. These types of surfaces are usually tuned towards the vast diversity of chemical and structural features that describe molecules derived from natural products. We further limited the study to two basic stationary phases: (1) non-resonance π-stationary phases, i.e. the cyano phase and (2) resonance π-stationary phases, i.e. phenyl type phases, of which we tested two: the phenyl-hexyl phase, that consists only of a phenyl ring tethered to the silica *via* a six carbon alkyl chain, and the pentafluorophenyl phase, which represents a modified phenyl moiety, of increased polarity in the F–C bonds, and has enhanced hydrogen bonding capabilities. Included in this study was a selectivity test undertaken on a Synergi Hydro-RP phase, giving the opportunity to assess the importance of the polar end-capping.

The chromatograms in Fig. 1(a–e) show the unidimensional separations of the espresso coffee on each of the five columns (including the Synergi Hydro-RP (d) and the C18 stationary phases (e)). The mobile phase in each case was a gradient of 100% aqueous to 100% methanol. Changes in selectivity were apparent on each column, but because the peak capacity was exceeded, selectivity changes were difficult to assess. In all cases, except on the Synergi Hydro-RP phase, the separation was essentially bimodal in distribution, as indicated by the dotted line separating the two distinct distributions. More information regarding the nature of the selectivity differences with respect to the C18 phase can be seen in the two-dimensional surface plots illustrated in Fig. 2(a–d). In each case the C18 phase was used in the second dimension, hence the change in peak displacement reflects the nature of the selectivity change in the first dimension. These surface plots clearly show that there were significant differences in the retention behaviour of the solutes undergoing migration through the 2D system.

Table 1
GAFA calculations for the 2DHPLC separations and in each of the quadrants.

System	N	Correlation	β	n_p	%Usage
Cyano (total)	138	0.742	42.1	1674	55.8
Cyano (Q1)	22	0.097	84.5	2665	95.2
Cyano (Q2)	54	0.457	62.8	2124	75.8
Cyano (Q3)	62	0.802	36.7	1397	49.9
Phenyl-hexyl (total)	105	0.908	24.8	1299	36.1
Phenyl-hexyl (Q1)	37	0.282	73.6	3081	85.6
Phenyl-hexyl (Q2)	13	−0.356	69.1	2937	81.6
Phenyl-hexyl (Q3)	53	0.893	26.8	1386	38.5
Pentafluoro-phenyl (total)	94	0.945	19.1	1036	28.8
Pentafluoro-phenyl (Q1)	37	0.079	85.5	3458	96.0
Pentafluoro-phenyl (Q2)	5	−0.572	55.1	2469	68.6
Pentafluoro-phenyl (Q3)	52	0.917	23.6	1243	34.5
Synergi polar-RP Hydro (total)	71	0.941	19.8	1072	29.8
Synergi polar-RP Hydro (Q1)	30	0.172	80.1	3288	91.3
Synergi polar-RP Hydro (Q2)	–	–	–	–	–
Synergi polar-RP Hydro (Q3)	39	0.871	29.5	1500	41.7

In order to assess qualitatively and quantitatively the separation power of the two-dimensional systems, the number (N) and two-dimensional retention times of eluting peaks were determined and then a geometric approach to factor analysis (GAFA) was applied to measure the *correlation* between each dimension, the spreading angle (β), the practical peak capacity (n_p) and percent usage of the separation space [23]. The data in Table 1 depicts numerically the changes that have occurred as a result of stationary phase selectivity. Overall, each of these systems shows relatively high correlation to that of the C18 phase, which is not unexpected since all coupled systems were RP-RP. However, there are distinct regions whereby specific systems would out-perform another system for certain sample components. Therefore, in order to more specifically detail the selectivity differences that were occurring, we have undertaken an extensive assessment of regional selectivity. To do that we divided the two-dimensional separation plane into quadrants, essentially consistent with the bimodal nature of the unidimensional separation. That is, each quadrant represents half the separation period from each dimension. We then measured the number of components in each region and undertook the GAFA assessment of each coupled region. This is illustrated graphically in Fig. 3, which shows retention time scatter plots for the location of

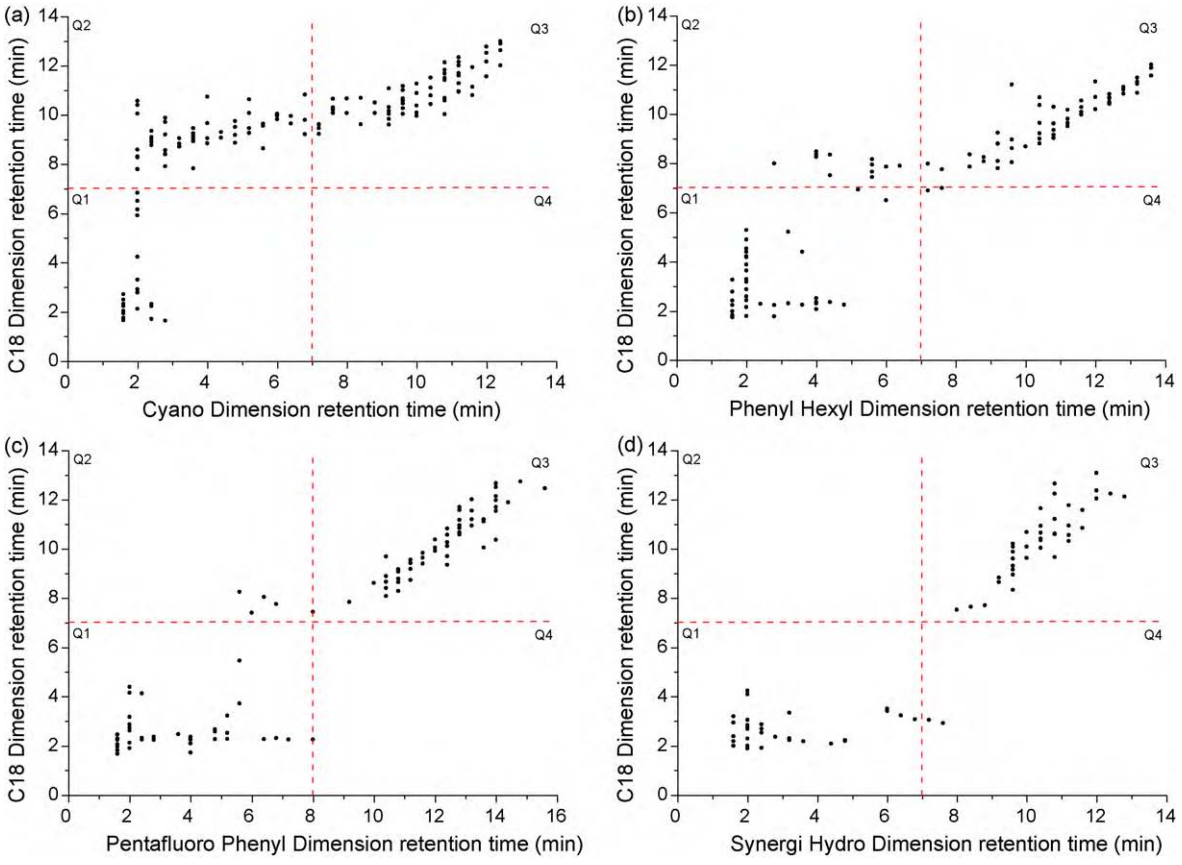

Fig. 3. Scatter plots for the 2DHPLC separations with (a) cyano, (b) phenyl-hexyl, (c) pentafluoro-phenyl and (d) Synergi Hydro-C18 first dimension columns. The quadrants are defined by the red dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

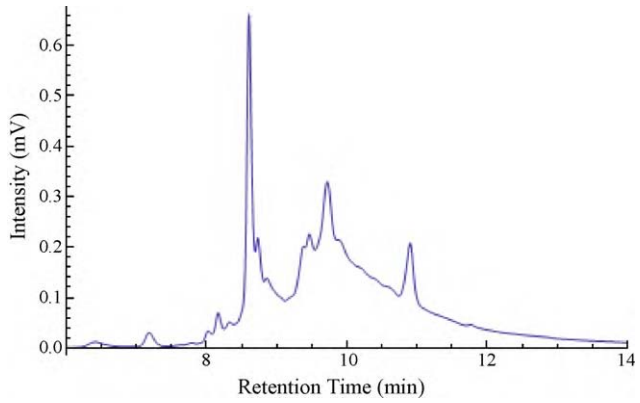

Fig. 4. Heart-cut segment separation of Ristretto café espresso on C18 phase at 3.2 min.

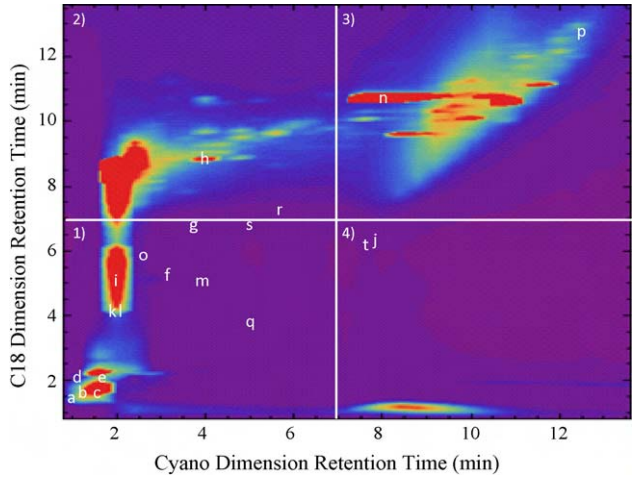

Fig. 5. Components identified in the Cyano/C18 system: Note, for the purposes of illustration the location of the components on the 2D plot represents only the generalised location, and not the extact 2D retention time: (a) caffeic acid, (b) malic acid, (c) quinic acid, (d) fumaric acid, (e) catechin, (f) a procyanidin dimer, (g) ferloylquinic acid, (h) ferulic acid, (i) 3-(4,5)-*o*-caffeoylquinic acid, (j) 3,4-dicaffeoylquinic acid, (k) trigonelline, (l) nicotinic acid, (m) sucrose, (n) caffeine, (o) caffeoylquinic acid, (p) rutin, (q) acetylated hexose based oligosaccharide, (r) oligosaccharide containing anhydrohexose, (s) acetylformoin hexose based oligosaccharide, (t) caffeoylshikimic acid, and (u) nicotinamide.

peak maxima in each of the four coupled 2D systems. The results from the GAFA for each system, and the four quadrants within each system are detailed in Table 1.

3.1. Qualitative assessment of the selectivity changes

3.1.1. Cyano phase

Quadrant 1: The peaks eluting from the cyano column in quadrant 1 (Q1) do so with very little retention on the stationary phase in the first dimension. However, these components display substantial variability in retention across the C18 phase, as shown by the separation on the C18 column for the heart-cut fraction at 3.2 min on the cyano column (Fig. 4). Hence these compounds have a wide range in polarity. This aspect of the two-dimensional retention behaviour indicates that these compounds have limited interaction with the non-resonance π-electrons or the dipole–dipole moment on the cyano phase, and separation in the second dimension is based on their hydrophobicity/methylene selectivity. Mass spectral analysis of compounds fractionated from this region of the chromatographic separation verified that these compounds were highly hydroxylated and in some instances were low molecular weight carboxylic or phenolic acids and monomeric flavan-3-ols. Within this quadrant, retention of the acids and monomeric flavan-3-ols in the second dimension increased with the aliphatic chain length (i.e. diCQA, di-procianidins) Alkaloids, such as nicotinic acid, nicotinamide and trigonelline (having cyclic amino rings) were observed to elute in this quadrant (Fig. 5).

Quadrant 2: The peaks in quadrant 2 (Q2) show a great deal of variability in their retention across the cyano phase, but limited variation on the C18 phase. They are therefore compounds of similar hydrophobicity, with little change in the nature of the carbon structure. Separation on the cyano phase is, however, obtained because of their selective interaction with the non-resonance π-electrons, and this indicates significant changes in the degree of functionalisation. Mass spectral analysis of the components collected from this region of the separation indicates that some of these compounds contain non-polar substituents, such as, the methoxy group in ferulic acid, which contributes to greater retention on the C18 dimension, but only slightly increased retention on the cyano stationary phase in the first dimension (Fig. 5).

Quadrant 3: The peaks in this quadrant (Q3) show discrimination in their retention on both phases. Therefore, these are compounds with variation in their carbon nature and in their degree of functionality. Mass spectral analysis confirmed the presence of more complex alkaloids, such as caffeine, which is the dominating compound in Q3, and polyphenols, such as rutin. The structures of these types of compounds are consistent with the observation of increasing solute-stationary phase interactions on these two phases. At this point in time we have not fully evaluated the nature of compounds eluting in this zone, as the complexity of the analysis is substantial. Having said that, we suspect that these compounds may be melanoidins, higher molecular weight polymeric species with a polydisperse structure containing nitrogen, carbohydrates, amino acids and phenolics [24].

Quadrant 4: No UV absorbing compounds were observed to elute in this region, indicating that there were likely to be few compounds that have highly polar or π-functional groups with a limited degree of carbon backbone (two components were detected by MS).

3.1.2. Phenyl-hexyl phase

Quadrant 1: In contrast to the cyano phase, the compounds eluting in Q1 were more highly retained on the PH phase. This suggests that these compounds had good interaction with the resonance π-electrons of the stationary phase surface. Again there was a general increase in retention of these compounds on the C18 phase, consistent with there being a significant change in polarity of these molecules. Mass spectral analysis verified that the same types of compounds that eluted in Q1 on the cyano system, also elute in Q1 on this system. However, for compounds, such as caffeic acid, retention on the PH phase increased considerably (Fig. 6), indicating the role of the resonance π–π selective interactions. Also of note is the retention of the compounds such as the pyridine derivative trigonelline, which increased on the PH phase (5 min retention time), in comparison to the cyano phase (2 min retention time).

Quadrant 2: Fewer compounds were observed to elute in this region than in the cyano system. This is consistent with two aspects of the separation: (1) the higher degree of correlation between both dimensions reduced the overall number of peaks detected and hence a greater degree of co-elution would be expected and (2) the greater degree of retention on the PH column resulted in the peaks that eluted in Q2 on the cyano system, now eluting in Q3 on the PH system (see later details in Table 1). This greater degree of retention is primarily a result of the increased hydrophobicity of the stationary phase due to the six member alkyl chain tethering the phenyl ring to the surface of the silica. There was also discrimination between species as a result of selective interaction with the resonance π-electrons.

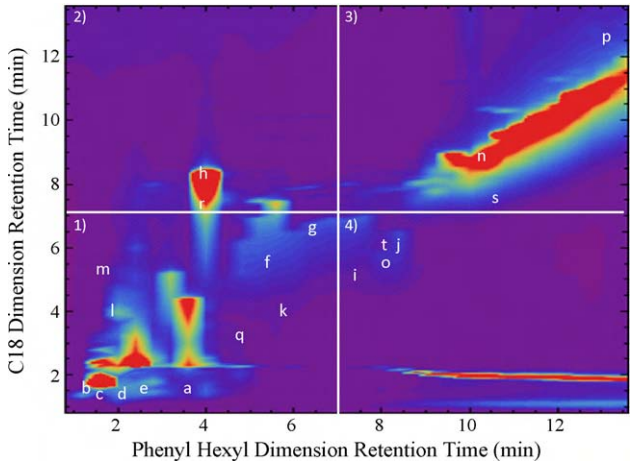

Fig. 6. Components identified in the phenyl-hexyl/C18 system: (a) caffeic acid, (b) malic acid, (c) quinic acid, (d) fumaric acid, (e) catechin, (f) a procyanidin dimer, (g) ferloylquinic acid, (h) ferulic acid, (i) 3-(4,5)-*o*-caffeoylquinic acid, (j) 3,4-dicaffeoylquinic acid, (k) trigonelline, (l) nicotinic acid, (m) sucrose, (n) caffeine, (o) caffeoylquinic acid, (p) rutin, (q) acetylated hexose based oligosaccharide, (r) oligosaccharide containing anhydrohexose, (s) acetylformoin hexose based oligosaccharide, (t) caffeoylshikimic acid, and (u) nicotinamide.

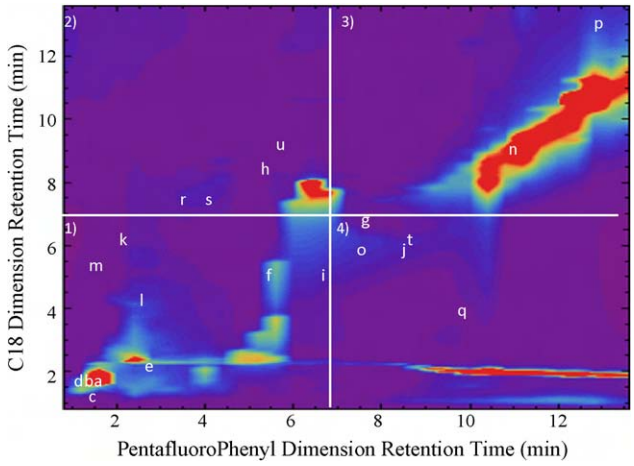

Fig. 7. Components identified in the pentafluorophenyl/C18 system: (a) caffeic acid, (b) malic acid, (c) quinic acid, (d) fumaric acid, (e) catechin, (f) a procyanidin dimer, (g) ferloylquinic acid, (h) ferulic acid, (i) 3-(4,5)-*o*-caffeoylquinic acid, (j) 3,4-dicaffeoylquinic acid, (k) trigonelline, (l) nicotinic acid, (m) sucrose, (n) caffeine, (o) caffeoylquinic acid, (p) rutin, (q) acetylated hexose based oligosaccharide, (r) oligosaccharide containing anhydrohexose, (s) acetylformoin hexose based oligosaccharide, (t) caffeoylshikimic acid, and (u) nicotinamide.

Quadrant 3: The most number of compounds for this system were observed in this quadrant. This is consistent with the differences in the aforementioned behaviours between the cyano phase and the PH phase, as the more hydrophobic species were retained to a greater extent on the PH column than the CN column, hence moving a significant portion of the compounds from Q2 to Q3. This supports the notion that these compounds display substantial variation in both their carbon backbone and likely their aromaticity. Mass spectral data verifies that in the third quadrant most of the eluted compounds were of hydrophobic character such as caffeine and sugars with non-polar substituents (rutin).

Quadrant 4: Two UV absorbing components (4 by MS) bordered the intersection of Q2, Q3 and Q4. There was, however, insufficient information to deduce whether these components were able to interact with the π-electrons, or whether they were in fact simply moderately polar. Nevertheless we were able to verify by mass spectrometry, that the polar 3(4,5)-*o*-caffeoylquinic acid (CGA) eluted in quadrant 4.

3.1.3. Pentafluorophenyl phase

Quadrant 1: Even greater retention of the components eluting in Q1 was observed on the PFP phase in comparison to the PH and CN phases, indicating perhaps that these components could undergo substantial hydrogen bonding. MS analysis revealed that the compounds eluting in this region were similar to those in PH and CN phases, but their retention was increased on the PFP dimension (Fig. 7). These compounds were either moderately polar nitrogen containing alkaloids, where the overall solute hydrophobicity played a role towards to PFPs discriminative retention or polar compounds with aromatic rings showing that π–π interactions were of major importance on PFP phase. This allowed the essentially unretained species on the more polar CN phase to be more strongly retained on PFP phase.

Quadrant 2: Only five components eluted in this region, indicative of the fact that there was greater retention on the PFP phase and thus the more hydrophobic components that could interact with either the resonance π-electrons, or those that could undergo hydrogen bonding were thus retained more on the PFP phase resulting in an increased number of components (as a function of the total number of components) eluting in Q3. In Q2, ferulic acid was identified, the methoxy moiety of which resulted in its higher retention in both PFP and C18 dimensions (Fig. 7).

Quadrant 3: The scatter of data points was highly correlated in Q3, perhaps indicating that the dominate aspect of retention for these species in this system was related to solute hydrophobicity. The mass spectral analysis confirmed the presence of high molecular weight sugar adducts, with non-polar substituents and caffeine.

Quadrant 4: No UV absorbing components eluted in Q4, but five components were detected by MS.

3.1.4. Synergi Hydro-RP phase

Quadrant 1: Greater retention of the components was observed in Q1 on this stationary phase. Of all the four systems tested, this combination yielded the least expression across the C18 dimension (see for example Fig. 2d), indicating these components were the polar species, undergoing interaction (likely hydrogen bonding) with the polar end-capping aspect of the stationary phase.

Quadrant 2: No components eluted in Q2.

Quadrant 3: Strong correlation was observed between the C18 and Synergi Hydro-RP phase in Q3. This is not surprising as both phases are C18 columns, and the more polar species showed their difference in interactions between the C18 and polar end capped C18 in their elution behaviour in Q1.

Quadrant 4: Two compounds eluted here, indicating the dominance of the C18 aspect of the stationary phase in comparison to the hydrophobicity, or the limited number of components able to interact with the polar end-capping.

Due to the insufficient separation selectivity differences between the Synergi Hydro-RP and C18 phases, further MS analysis to elucidate the main functionality of eluting compounds distributed in this particular system was not undertaken.

3.2. Quantitative assessment of selectivity changes

3.2.1. Overall system performance

The total number of detected peaks in each of the four coupled systems is given in Table 1. The most number of peaks was observed to elute from the cyano system, consistent with this system yielding the least correlation between dimensions. The number of detected peaks eluting from the π selective stationary phases decreased with

increasing correlation. In all four of these separations, only one system showed peaks eluting in Q4 (Synergi Hydro-RP coupled to the C18), but this was limited to just two peaks. For the most part, components were scattered throughout the other three quadrants, with the exception, again, of the Synergi Hydro-RP phase where no components were observed to elute in Q2.

The least correlated system was the cyano system (0.74), followed by the phenyl-hexyl system (0.91). The pentafluorophenyl phase and the Synergi Hydro-RP phase were almost exactly the same, with respect to total system performance with correlations of 0.945 and 0.941 respectively. The practical peak capacity of the cyano phase was 1674 (55% usage), almost 300 peaks greater than the next 'best' system (phenyl-hexyl phase) with 1299 peaks (36% usage).

Assessment of the separation performance of each of these systems based on a global performance measure, however, does not illustrate important localised performance measures. In order to assess the localised performance, GAFA was applied to the elution of the components in each of the elution quadrants within the 2D separation plane.

3.2.2. Localised system performance

Quadrant 1: Each of the four stationary phases that were coupled to the C18 phase showed almost orthogonal retention behaviour to the C18 phase with correlation coefficients between 0.079 (PFP) to 0.282 (PH). Even the Synergi Hydro-RP phase showed considerable diverse retention behaviour to the C18 ($c = 0.172$). The cyano phase was correlated at 0.097. All four phases showed greater than 85% utilisation of the separation space, with the least correlated phase (PFP) utilising 96%. Despite the relatively high degree of space utilisation that was observed for the cyano phase, the detection of only 22 components compared to 37 on the PFP phase, suggests a number of multiplets in this region of the separation. Overall, in Q1 small phenolic acids and alkaloids were observed to elute, irrespective of the stationary phase, but with retentivity generally increasing in the order CN < PH < PFP.

Quadrant 2: The cyano phase presented the greatest utilisation of this separation region, with a total of 54 components being observed. In comparison, only 13 and 5 components were observed to elute in this quadrant on the PH and PFP phases respectively. No bands were observed to elute in this region from the Synergi Hydro-RP phase. Correlation between the cyano dimension and the C18 dimension increased in this quadrant, moving from 0.097 in Q1 to 0.457 in Q2, with an overall percentage utilisation of separation space in this dimension being 75.8% of the theoretical peak capacity. In contrast, both the PH and the PFP phases showed inverse correlation with the C18 phase in Q2, although this was tested on fewer components (5 on the PFP, but 13 on the PH phase and still significant). In Q2 mostly compounds of moderated polarity, such as ferulic acid and feruloylquinic acids, have been determined.

Quadrant 3: All four columns showed the greatest number of peaks (with respect to their total number separated) eluting in Q3. Likewise, correlation between each phase and the C18 phase was at its greatest in this quadrant, with correlation values between 0.802 (cyano) to 0.917 (PFP). Components eluting from the Synergi Hydro-RP and the two phenyl phases in particular showed strong alignment of the main diagonal in this quadrant. Hence, the percent usage of the separation space decreased to as little as 35% on the PFP phase, and 50% on the most divergent phase (cyano). It is not surprising that correlation is greatest in Q3 since compounds that elute in this region are the least polar compounds in the sample and their retention more than likely reflects their hydrophobicity. In Q3, caffeine was the most abundant species, but it is likely that other nitrogen containing hydrophobic molecules are present.

Quadrant 4: Selectivity was not assessed in Q4 since there were too few compounds in any of the systems to gain any degree of useful information.

4. Overview

Without doubt, the cyano phase showed the greatest overall selectivity difference with respect to the C18 phase. Hence, applications in the 2D analysis of espresso coffee would be better served utilising this combination than any of the other three if the C18 phase were to remain in the second dimension. However, it is important to note that there was significant selectivity differences observed between each of the phases when the data analysis was directed to more specific regions of the separation space. For example, the PFP showed much greater separation potential for the compounds that eluted in the Q1 region compared to the PH phase, and in fact in this region the PFP phase was marginally more powerful than the cyano phase. The limiting factor of the PFP phase and PH phase, with respect to providing greater separation performance in comparison to the cyano phase was that these phases were highly correlated in Q3 (i.e. the hydrophobic nature of the stationary phase dominated retention). Therefore this limited the number of components that could be displaced into Q3, which decreased the overall percentage utilisation of the 2D separation plane.

Another interesting factor derived from this study is that despite the Synergi Hydro-RP phase being predominately C18, substantial selectivity differences were observed relative to the C18 phase, although not to the same extent as the π selective phases, as displacement was largely limited to Q1 and Q3. Furthermore, fluorine substitution on the phenyl phase altered retention behaviour, presumable due to hydrogen bonding.

Importantly, it is worth noting that almost orthogonal retention behaviour was observed between each of the four phases in combination with the C18 phase in at least one quadrant. Furthermore, reverse correlation against the C18 phase was observed for the two phenyl phases in Q2. However, in all phase combinations the degree of correlation was higher for the total of all three quadrants when combined than when looking at a localised (Q1, Q2) correlation factor. This implies, that (a) it may be impossible to obtain a two-dimensional system that yields orthogonal selectivity behaviour for samples of complex natural origin containing a large number of analytes and (b) great care must be exercised if model compounds are to be used to assess selectivity differences across different coupled systems [11], because clearly these results show that the measure of orthogonality depends on the nature of the sample, with respect to the separation environment.

Finally, as to which coupled system would be the best for the analysis of coffee, it depends largely on the objective of the study. In this series of investigations we are primarily interested in the identification and isolation of antioxidants in coffee. For that reason, a companion study has been undertaken that employs chemiluminescence detection in the screening of coffee samples separated by 2DHPLC employing the cyano-C18 combination. Only after the separation displacement of the antioxidants has been determined can the optimal separation system be determined, that satisfies the goals of this experiment.

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