A simple approach for reversed phase column comparisons via the Tanaka test

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ABSTRACT

The Tanaka test is a classical characterization scheme designed in 1989 for highlighting the main reversed phase attributes of C18 columns: hydrophobicity, shape/steric selectivity, hydrogen bonding, ion exchange (pH < 7 and pH > 7 environments). In this short communication, we present it in a simplified manner that requires basic HPLC and spreadsheet operations; encouraging analysts of all levels of expertise to begin capturing an in-house library of column comparison knowledge based on their laboratory's unique set of phases and previously developed separations. This approach does not compete, nor replace existing techniques such as quantitative structure-retention relationship (QSSR) models to aid column selection. It is simply an alternative or additional aid to support column selection decisions that is easily accessible and implementable.

1. Introduction

The original Tanaka test and the visual representation via six axes radar plots were first developed in 1989 to characterize the retention and selectivity differences, between reversed phase C18 fully porous particle (FPP) columns [1]. The initial study verified that measurements from five isocratic injections could provide insights about the particles that are associated to the main characteristics that dictate selectivity behavior: amount of alkyl chains, hydrophobicity, shape selectivity, hydrogen bonding capacity and ion exchange capacity in two different environments (pH < 3 and pH > 7).

Six axes radar plots were originally developed as a tool to distinguish differences for each critical parameter that dictates the key interactions for reversed-phase columns (Table 1). Differences which may be associated to the synthesis of the C18 stationary phase such as: the type of silane used, acid pre-treatment of the silica, endcapping, surface coverages, surface area and pore size. In the original Tanaka et al. study, destructive characterization techniques verified that the chromatographic assays could determine synthesis differences between various C18 columns manufactured. Surface coverage, and amount of silanols were the main synthesis characteristics that could be associated to the visually different radar plots [1]. Furthermore the Tanaka test has been used compare different novel and manufactured reversed phase columns of different formats [2–4].

The robustness of the Tanaka test was studied in 2005, and certain method parameters were defined in order to utilize the test for identifying batch to batch selectivity differences between manufactured C18 columns [5]. In 2016, the Tanaka test was extended to superficially porous particle (SPP) technologies, and in particular, the utility of SPP technology in pharmaceutical separations, as batch to batch reproducibility is a critical factor in pharmaceutical analyses [6].

The Tanaka test is most beneficial for laboratories synthesizing novel stationary phases. However, the utility may also serve a greater purpose accessible for all laboratories, to create a data-base of in-house knowledge that represents the retention and selectivity behavior of the laboratory's and/or analyst's unique set of columns. As columns start to accumulate within the tool-box, similarities and differences may be visualized and aid column selection and method development decisions.

Previous studies have compared other stationary phases of different column formats via comprehensive strategies [7–10]. Often every lab has its own unique set of reversed phase columns. The focus is not on the column, but how can an analyst make column comparisons and selection decisions based on their own resources and in an easily adoptable fashion – without high level expertise in chromatography, nor modelling. Comparing and selecting a column can either rely on a literature search, on a marketing sales representative, and/or running experiments. These are all very time consuming and there does not yet exist a universal approach [9]. In this paper, we present the Tanaka Test in a simplified manner, where its utility can translate across different analytical laboratories with basic HPLC and spreadsheet operations for

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Table 1
The original Tanaka test characterization scheme [1].

Test	Property tested	Associated stationary phase characteristics	Isocratic mobile phase conditions	Chromatographic Measurements
A	Amount of alkyl chains	Silica surface area, and surface coverage	80% Methanol and 20% Water	k amylbenzene
B	Hydrophobicity	Surface coverage	Same as test 1	k amylbenzene/ k butylbenzene
C	Shape/Steric selectivity	Functionality of the silane, and surface coverage	Same as test 1	k triphenylene/ k o-terphenyl
D	Hydrogen bonding capacity	Amount of silanols, endcapping, and surface coverage	30% Methanol and 70% Water	k caffeine/ k phenol
E	Ion exchange capacity(pH > 7)	Amount of silanols and ion exchange sites	30% Methanol and 70% Water (aqueous portion: 0.02 M phosphate buffer, pH 7.6)	k benzylamine/ k phenol
F	Ion exchange capacity(pH < 3)	Amount of silanols and ion exchange sites at pH 3, and silica pretreatment	30% Methanol and 70% Water (aqueous portion: 0.02 M phosphate buffer, pH 2.7)	k amylbenzene/ k butylbenzene

column selectivity comparisons. The Tanaka test need not be limited to batch to batch studies, or stationary phase column manufacturing and/ or development laboratories. The information from the Tanaka test can be used to aid analysts with column selection and method development decisions based upon their own unique set of reversed phase stationary phases.
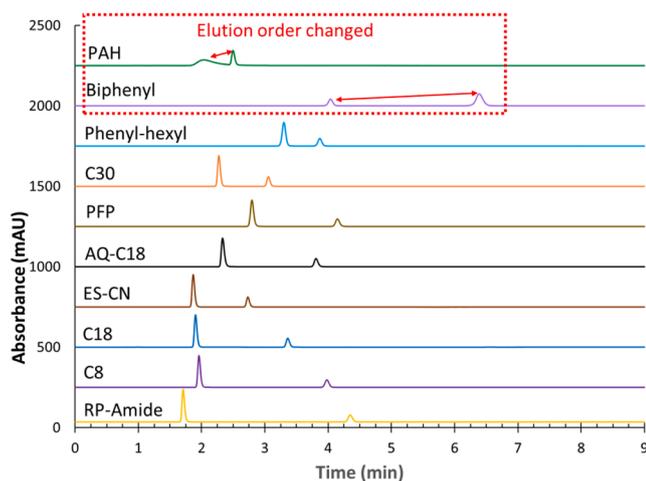
Fig. 1. Illustrating the importance to capture elution order changes experienced for the PAH and biphenyl stationary phase (green and light purple trace) relative to the eight different stationary phases for the test of hydrogen bonding capacity.

2. Experimental

2.1. Chemicals

All solvents used were HPLC grade. Methanol, acetonitrile, mobile phase additives, and individual standards were obtained from Milli-poreSigma (St. Louis, MO).

2.2. Chromatography columns

Ten different reversed phase stationary phases with the following properties were tested: 2.1 × 100 mm column format, 2.7 μm particle diameter (d_p), 90 Å (unless specified otherwise) superficially porous particle packed columns. The stationary phases characterized in this study were as follows: C18 (six columns synthesized from six different batches), C8, C30 (160 Å), AQ-C18, phenyl-hexyl, polyaromatic hydrocarbon (PAH), pentafluorophenyl (PFP), biphenyl, RP-Amide and cyano (CN) HALO® columns from Advanced Materials Technology, Inc. (Wilmington, DE).

2.3. Instrumentation

All experiments were run on a Shimadzu Nexera HPLC instrument (Columbia, MD) using a UV diode array detector (1 μL flow cell) set at a wavelength of 254 nm, and LabSolutions software (Shimadzu).

2.4. Chromatographic conditions

Mobile phases for the Tanaka test were prepared gravimetrically and the pH meter was calibrated before each use for the aqueous fraction of the mobile phases required for the ion exchange experiments. The columns were initially equilibrated with the minimum of 20 columns for each change in the mobile phase environment. The dead time of the column (t_0) was measured with the use of the minor disturbance method [11]. The isocratic runs and conditions were performed as listed in Table 1 at the flow rate of 200 μL/min, 40 °C, with an injection volume of 0.5 μL, injections performed in triplicate. The use of the retention time of uracil was used to confirm t_0 for cases when the minor disturbance is not clearly observed. The flow rate can be adjusted to higher velocities for time conscious labs. Injection volumes and velocities may be adjusted depending on the column's dimensions. Six batches of C18 columns were tested to illustrate the validity of the Tanaka test to visually compare similar and differences in RP attributes and batch to batch differences.

The polycyclic aromatic hydrocarbons (PAH) separation conditions were as follows: mobile phase A – water; mobile phase B – acetonitrile; gradient conditions: 50 to 100% B in 4 min, 100% B held for 1 additional minute and then brought to initial conditions in 0.1 min; injection volume: 2 μL; temperature 30 °C, column format: 90 Å, 2.7 μm d_p, 4.6 × 50 mm. The 18 PAHs eluted in the following order for the PAH phase: naphthalene, acenaphthylene, 1-methylnaphthalene, 2-methylnaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b] fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h] anthracene, benzo[g,h,i]perylene and indeno[1,2,3-c,d]pyrene.

The anticoagulant separation conditions were as follows: mobile phase A – 0.1% formic acid in water adjusted to pH = 2.6; mobile phase B – 50/50 acetonitrile/methanol; isocratic conditions: 40/60-A/B; injection volume: 1 μL; 254 nm; temperature 45 °C; column format: 90 Å, 2.7 μm d_p, 4.6 × 50 mm.

The basic compounds separation conditions were as follows: mobile phase A – ammonium formate buffer, 0.01 M, pH = 3; mobile phase B – acetonitrile; isocratic conditions: 5/95-A/B; injection volume: 1 μL; 254 nm; temperature 30 °C; column format: 90 Å, 2.7 μm d_p, 4.6 × 50 mm.
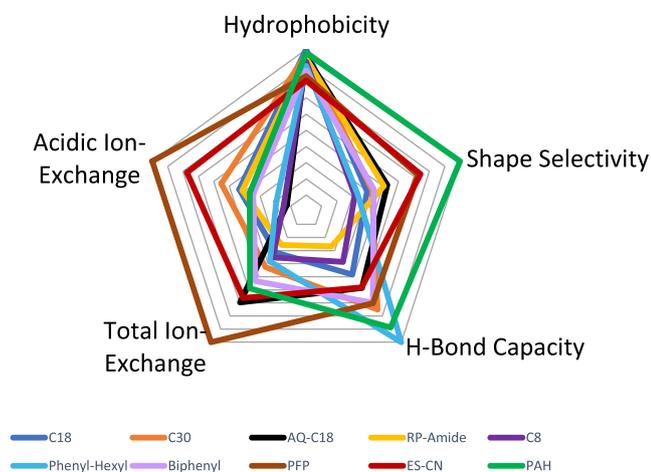
Fig. 2. Normalized radar plot for ten stationary phases of differing selectivities.

2.5. Data analysis

Measurements are based on retention factors (k) = (retention time of your analyte of interest $(t_R–t_0)/t_0$. Furthermore, the selectivity factor (α), the ratio of k for two analytes of interest $(\alpha = k$ of the most retained analyte of interest/k of the least retained analyte of interest).

The six properties that influence retention and selectivity for reversed phase liquid chromatography separations listed in Table 1, serve the basis of a six axes spider/radar plot. Tests A and B can be combined in one injection (see Table 1). The 'Euerby-modified' Tanaka plots exclude the amount of alkyl chains axis, as the selectivity information of the hydrophobicity parameter contains this information [2]. For example, a stationary phase that is less retentive, most likely has less alkyl chains/ligands bonded to the surface and is less hydrophobic.

3. Results

3.1. Initial inspection of chromatograms

The importance of scanning the chromatographic peaks for each of the five isocratic tests is to firstly identify any elution order changes. For example, in Fig. 1, it is clear that the first and second column/separation performed on the PAH (green trace) and biphenyl (light purple trace) stationary phases, both experienced an elution order change relative to the other tested columns.

Essentially the distance between two peaks determines the selectivity factor and elution order changes complicate the comparison on the five axes plot by skewing the scale of that metric. Therefore, strictly for comparative purposes, the reciprocal value of the elution order change is taken.

Furthermore, it is important to inspect the peak shapes for preliminary information on stationary phase attributes that clearly differentiate amongst the stationary phases evaluated. For example, in Fig. 1 the peak shape behavior of the PAH phase highlight that compared to the nine other stationary phases there is a clear difference in either/combination of the amount of silanols, the endcapping procedure and/or the surface coverage.

It is up to the discretion of the analyst to gauge and compare the peak shape behavior as auxiliary information from the chromatograms. However, we highlight that these tests were designed to evaluate retention and selectivity behavior between various reversed phase columns, not column efficiency.

3.2. Constructing the radar plot

Once the elution order change results have been taken into account,
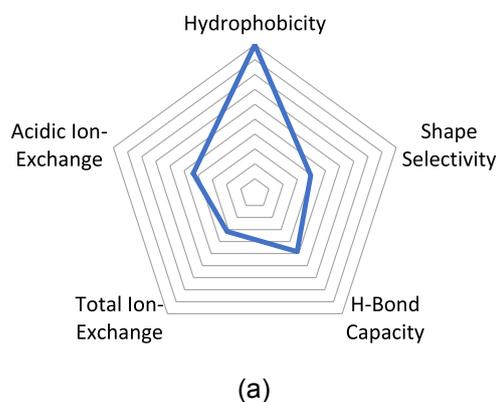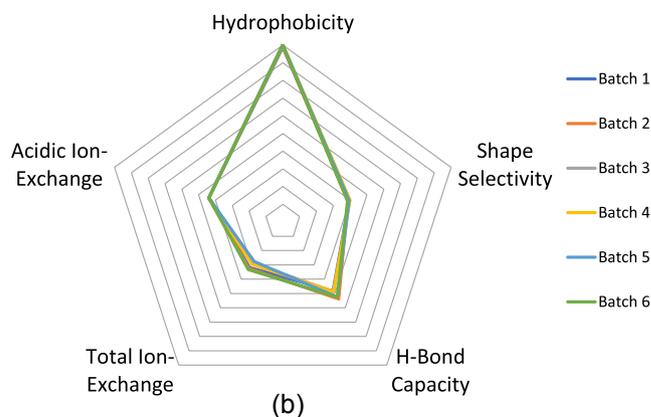
Fig. 3. a. The radar plot for the most hydrophobic stationary phase studied (C18 column). **b.** Comparison of six different batches of the C18 phase.

the radar plot can be constructed. A simple template is provided in the supplementary information spreadsheet S1 to compare seven different columns. The highest value for one axis may result in a relatively larger value than the other axes, and can heavily skew the radar plot. This complicates the visual process of identifying relative differences and similarities for the other axes. Hence, normalization is required for each axis. For a global comparison of different stationary phases, the spreadsheet provided in the supplementary information has included a function to search and divide all values by the largest value for each axis. In Fig. 2, the values for each individual axis have been normalized to the maximum value.

The graph depicted in Fig. 2 represented and compared ten different reversed phase stationary phases within our lab using Fused-Core®/superficially porous particle technology. The global comparisons are useful to visualize relative differences, similarities, and columns that exhibit the strongest characteristics for each parameter. While the plot for multiple different stationary phases can crowd the plot, removing traces is recommended to highlight comparative differences for different purposes and will be discussed in the following sections.

3.3. Hydrophobicity – column comparison and selection

A 'go to' C18 column is common for the majority of laboratories and serves as the starting point for screening or method development work. Whilst a universal stationary phase for all separations would be ideal, sample matrix complexity and target compounds are often subject to change. Testing various columns in the lab with the sample/standards of interest may at times be limited in terms of cost and availability. Hence, the radar plots can aid in column selection decisions and highlight other reversed-phase separation characteristics that may be exploited to provide an alternative separation to the laboratory's 'work-horse' C18

(a)

(b)
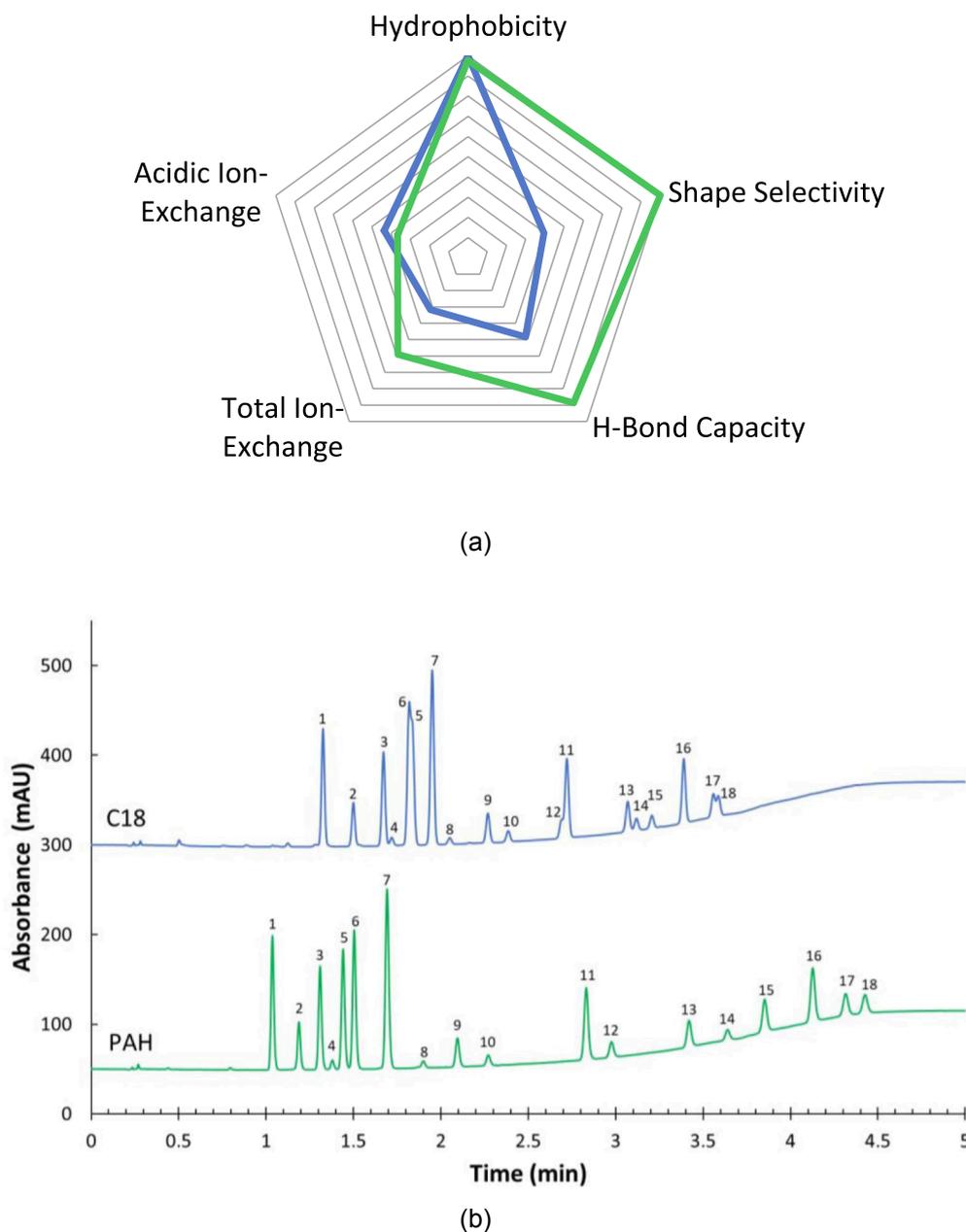
Fig. 4. a. The radar plots for the phase that exhibited the strongest shape selectivity, (PAH stationary phase) and the C18 phase (same key as Fig. 2). **b.** Chromatographic comparison for the separation of 18 polycyclic aromatic hydrocarbons (PAHs) requiring shape selectivity.

column.

Utilizing the radar plot it could be seen that the C18 retention behavior is mainly governed by hydrophobicity and may offer other attributes (Fig. 3a). Moreover, six batches of C18 columns were tested and visually compared in Fig. 3b to highlight similarities and differences for each of the RP attributes. The largest standard deviation of 0.01 for all the calculated selectivity factors resulted (inter-day analyses with fresh mobile phase preparation).

The following radar plots, along with an illustrative chromatogram or listed applications, represent the reversed phase range depicted in Fig. 2 for the column that achieved the largest value for that specific parameter/axis point, compared to the C18 work-horse phase to demonstrate our in-house column comparison and selection decisions.

3.4. Shape selectivity - column comparison and selection

The PAH stationary phase resulted with the highest shape selectivity

compared to all other nine columns (Fig. 2 and Fig. 4a). The PAH column is in fact a C18 bonded phase that was tailor designed for shape selectivity. The PAH stationary phase is not endcapped, and without knowing this piece of information, the radar plot together with the chromatograms in Fig. 1 and the insight from Table 1 could all be used to deduce that the amount of silanols are the main contributing characteristic of the phase. Note, due to the increased amount of silanol groups, such phases are not recommended for the separation of basic compounds.

The shape selective tailor-designed moiety was exploited for high throughput analyses of a shape selectivity separation problem represented by 18 PAH contaminants illustrated in Fig. 4b. The comparative chromatograms between the C18 and the 'PAH' (non-endcapped C18) stationary phases demonstrated when the 'workhorse' C18 column does not work. Referring to the radar plot a column selection decision can be achieved very quickly for a separation that has target analytes that are very similar to one another and differ in their shape.

(a)

(b)
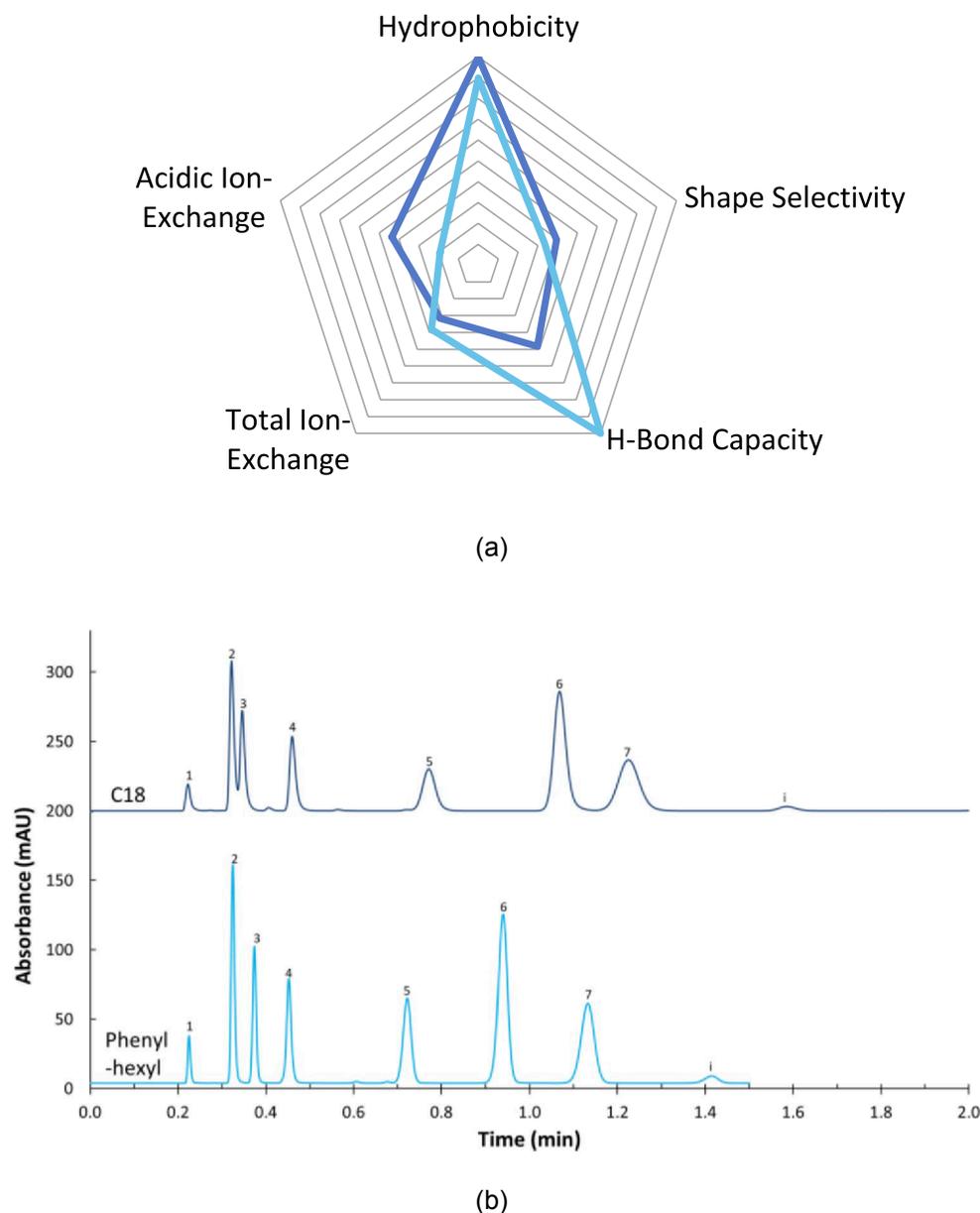
Fig. 5. a. The radar plots for the largest hydrogen bonding capacity phase (phenyl-hexyl stationary phase), and the C18 phase (same key as Fig. 2). **b.** Chromatographic comparison of the phenyl-hexyl separation of seven anticoagulants exploiting these differences compared to the C18 phase.

3.5. Hydrogen bonding - column comparison and selection

The phenyl-hexyl phase offers pi-pi interactions and displayed the highest H-bonding capacity compared to the other nine reversed phase columns evaluated (Fig. 2). It has significantly decreased acidic ion exchange/residual silanol group interactions and slightly higher total ion exchange (pH > 7) compared to the C18 (Fig. 5a). Hence, it has quite a significant amount of silanol groups that contribute to the interaction.

Applications that exploit these retention and selectivity characteristics of this phase include penicillins, anti-coagulants, and fluoroquinolone drugs. Fig. 5b. highlights a chromatographic comparison of exploiting the hydrogen bonding characteristics of the phenyl-hexyl phase for higher resolution separation of seven anticoagulants with the following elution order: uracil, 4-hydroxycoumarin, coumarin, 6-chloro-4-hydroxycoumarin, warfarin, coumatetralyl, coumachlor and an unidentified impurity; compared to the C18 phase. This section demonstrated how a quick decision could be made - exploiting the Tanaka radar plot for a fast visual aid based on in-house empirical results

to very quickly to identify which column exhibits the strongest hydrogen bonding characteristic and select that column for separations that require this attribute.

3.6. Ion exchange > pH7 and < pH7 - column comparison and selection

The PFP stationary phase behaved distinctly different to the C18 (Fig. 6a) and in the column comparison used in Fig. 6a it showed both the largest characteristics for total and acidic ion exchange. Additionally, a quick visual of the radar plot demonstrated that it differed in all characteristics compared to the 'workhorse' C18 of the lab. Moreover, this phase was utilized for analytes such as mycotoxin screening and tranquilizer separations that exploited the PFP's unique retention characteristics.

Due to the PFP's increased amount of ion exchange sites it has the versatility to be employed in both reversed phase and hydrophilic interaction liquid chromatography (HILIC) separations. Hence, in Fig. 6b. we illustrated the separation of six basic compounds with the

(a)

(b)
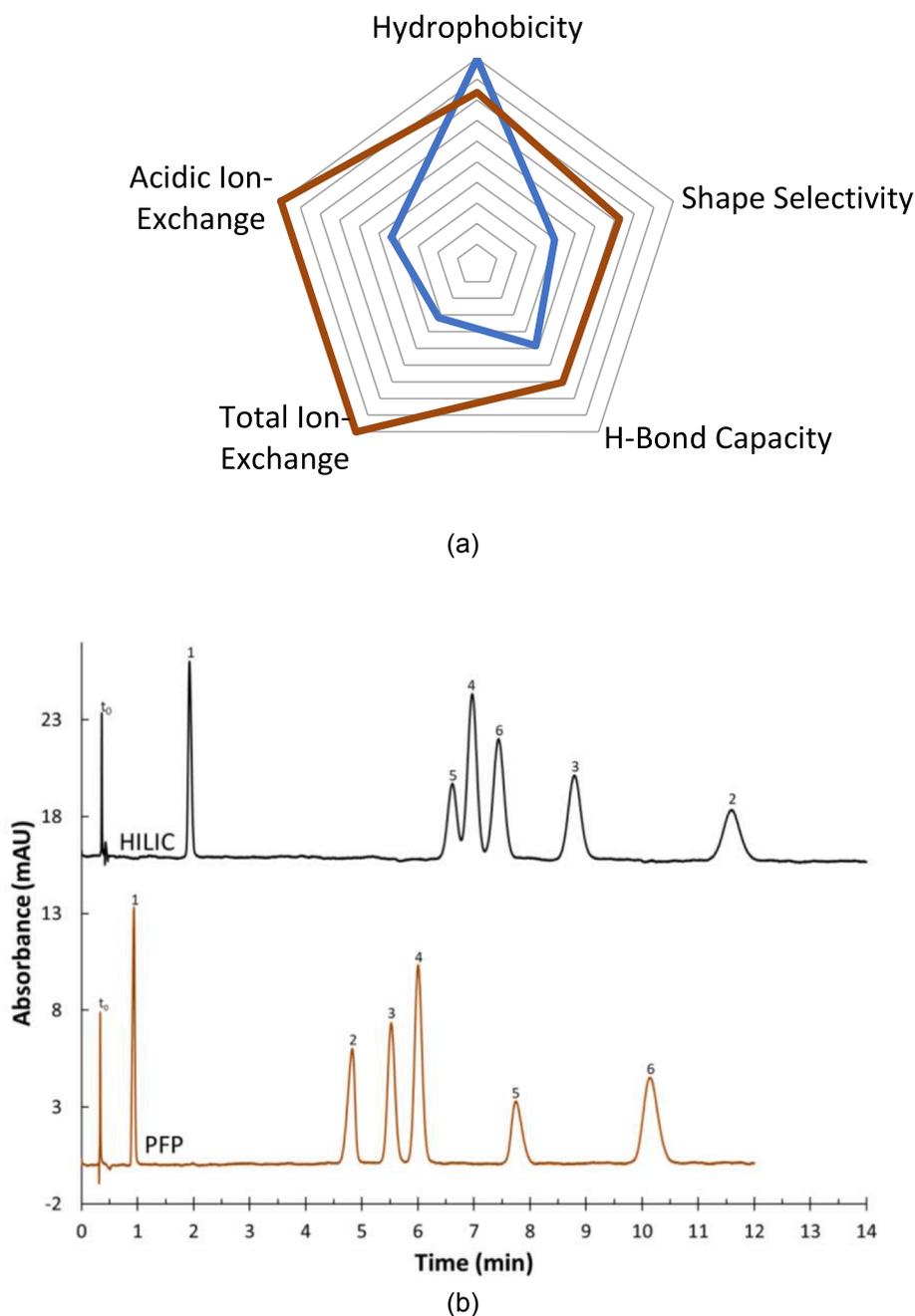
Fig. 6. a. The radar plots for the phase that had the strongest total and acidic ion-exchange characteristics (pentafluorophenyl (PFP) stationary phase), and the C18 phase (same key as Fig. 2). **b.** Chromatographic comparison of the PFP phase for the separation of basic drugs exploiting the ion-exchange capabilities compared to a HILIC column.

following elution order for the PFP column: trazadone, phenylephrine, procaine, amoxapine, propranolol, desipramine (Fig. 6b); in comparison to a HILIC column, under HILIC separation conditions.

It is recommended as the Tanaka test comparisons are constructed for in-house column comparison purposes, that a list of applications/ methods may also be captured (as shown in this study). This is especially useful for laboratories dealing with restricted/sensitive sample information. Thus, when a new separation problem arises, column screening/ method development decisions can begin with scanning the information captured in the laboratory's radar infographics accompanied with the listed analyte(s) or chemical groups successfully separated using the column's attributes.

Additional general conclusions of this brief communication include: since a universal approach to compare and select columns does not yet

exist, we demonstrated a simple approach that does not require a high level of chromatography expertise, nor computational modelling. This approach does not serve as a replacement of existing and comprehensive strategies to aid column comparison and selection decisions, but an alternative, easily implementable method that is strictly based on the laboratories' own set of columns.

4. Conclusion

In this short communication, we have taken the classical Tanaka test set of experiments developed in 1989, and have demonstrated step by step the utility to aid column comparisons accessible for analysts with basic HPLC and spreadsheet operations. Understanding the history and development of the Tanaka and 'Euerby-modified' test is important.

Whilst, the utility of the information to aid your own column selectivity 'in-house' knowledge based upon your own unique set of columns is invaluable. The five axis radar plots can easily be constructed and comparisons based on the laboratory's own unique inventory of different reversed phase columns can be made. A database can then be constructed that can aid column screening/selection for reversed phase separations that are mainly governed by one or a combination of hydrophobicity, shape selectivity, hydrogen bonding capacity, total and/ or acidic ion exchange.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105793.

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