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

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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

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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,