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A two-component post-column derivatisation method utilising reaction flow chromatography

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ABSTRACT

"Reaction flow (RF) chromatography" has recently emerged from exploiting a new column "Parallel Segmented Flow" (PSF) to post-column derivatisation (PCD) methods [1]. The main advantage RF has over conventional PCD methods is the elimination of large volume reaction coils, as mixing occurs in the PSF column outlet fitting design, therefore minimising peak broadening. In this investigation we take an existing PCD method for the detection of phenols and apply it to RF. The detection sensitivity of the RF method is discussed, with the results demonstrating that through careful control of the mobile phase and derivatisation reagent conditions the selectivity of the PCD reaction may target compounds other than phenols.

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1. Introduction

Reaction flow chromatography is a new separation technique involving post column derivatisation (PCD) with a newly designed HPLC column [1]—the reaction flow chromatography column (RF) [2–13]. RF allows for the reaction/mixing to occur inside the outlet end fitting of the column, which eliminates the need for reaction coils, hence minimizing the post-column dead volume, which for some assays may be as large or even greater than 500 μL [1,14]. Extra-column volumes with modern chromatography columns are deleterious to their performance [15]; hence RF is a promising approach for PCD reactions in a manner that does not compromise the separation efficiency gained by the column separation.

PCD methods allow for selective detection, and thus are advantageous for targeted analysis and/or to decrease sample complexity. A recent review by Zacharis and Tzanavaras highlights the history and application, as well as the instrumentation and chemistries involved [14]. They state that the chemistry involved in PCD reactions will be the driving force for its impact and application, and also instrumental improvements that support lower dead volumes and better mixing. In their concluding remarks and perspective they highlight the advantages of RF chromatography, emphasising the importance on maintaining column efficiency.

In our preliminary work on RF chromatography we tested the performance of post-column derivatisation for the analysis of antioxidants with diphenylpicrylhydrazyl radical (DPPH•) and compared its advantages to a conventional post column derivatisation approach (standard column + reaction coil) [1]. In our prior work, gains in column efficiency were apparent, since the dead volume was dramatically decreased, and in addition, gains in sensitivity were also apparent, in part, as a result of the decreased band broadening due to the decreased post-column dead volume [1].

The application of RF is so far new and largely untested, but shows great promise. The aim of this study is to modify an existing literature PCD method [16] that is stated to be selective for phenols and convert it to an RF approach. The importance of the PCD method chosen for this study is that it involves the addition of two post-column derivatising reagents, rather than just the one, as was the case for the antioxidant testing. As such, not only is this just the second PCD method developed on RF columns, it is the first one that utilises two post-column derivatisation reagents.

2. Experimental

2.1. Chromatography column

Hypersil GOLD reaction flow columns (150 × 4.6 mm, $d_p = 5$ μm) were supplied by ThermoFisher Scientific (Runcorn, Cheshire, United Kingdom). Further details of reaction flow columns can be found in reference [1].

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2.2. Chemicals

HPLC grade methanol was purchased from ThermoFisher Scientific (Scoresby, Victoria, Australia). Milli-Q water (18.2 MΩ cm^{-1}) was prepared in-house and filtered through a 0.2 μm filter. 4-Aminoantipyrene was manufactured by Acros Organics BVBA (Loughborough, Leicestershire, UK). Potassium ferrocyanide was purchased from AnalaR (Murarrie, QLD, Australia). Ammonium acetate, phenol, 4-methoxyphenol, *p*-cresol, tocopherol, toluene, propylbenzene, *sec*-butylbenzene, butylbenzene, pentylbenzene, hexylbenzene, heptylbenzene, octylbenzene, naphthalene, anthracene, chrysene, anisole, phenetole, caffeine, phenylalanine and benzamide were all purchased from Sigma Aldrich (Castle Hill, NSW, Australia). All materials were used as received. All mobile phases were prepared volumetrically and used without further filtration.

2.3. Equipment

All chromatographic experiments were conducted using a Shimadzu chromatographic system (Shimadzu, Rydalmere NSW, Australia) equipped with a Shimadzu LC-20ADvp quaternary pump for mobile phase delivery, and two Shimadzu LC10ADvp pumps for the delivery of both post-column derivatisation reagents. The system was also equipped with a Shimadzu SIL-10ADvp auto injector, Shimadzu SPD-M10Avp photo diode array detector and two inline degassing units for all mobile phase and post-column derivatisation reagent delivery lines.

2.4. Reagent and sample preparation

The ammonium acetate (0.1 M) buffer solution (adjusted to pH 9 with ammonia) was used to prepare the post-column derivatisation reagents: 1) the 4-aminoantiprine solution (150 mg/100 mL) and 2) the potassium ferricyanide solution (150 mg/100 mL in line with the procedure outlined by Bigley and Grob [16].

A stock calibration standard containing phenol, 4-methoxyphenol, *p*-cresol and tocopherol was prepared in 100% methanol using volumetric glassware. The calibration standard was quantitatively diluted to yield standards between 20 and 2000 ppm in the case of phenol and 4-methoxyphenol and 200 and 3200 ppm for *p*-cresol and tocopherol. These standards were analysed in duplicate, within a single day.

A laboratory test sample "test sample mixture" was prepared using a variety of compounds that included alkyl benzenes, polynuclear aromatic hydrocarbons (PAHs), anisole, phenetole, caffeine, phenylalanine and benzamide. The sample included phenol, 4-methoxyphenol, *p*-cresol, tocopherol, toluene, propylbenzene, *sec*-butylbenzene, pentylbenzene hexylbenzene, heptylbenzene, octylbenzene, naphthalene, anthracene, chrysene, anisole, phenetole, caffeine, phenylalanine and benzamide. This sample was made qualitatively in 100% methanol.

2.5. Chromatographic methods

The initial optimisation studies were conducted using phenol standards under isocratic conditions for the column separation using a mobile phase composition of 5% A and 95% B; where A refers to 100% water at pH 9 buffered with ammonium acetate (0.1 M) and B was 100% methanol. The HPLC flow rate was 1.0 mL/min. Although the pH of the mobile phase was relatively high, the stability of the column was maintained throughout the duration of the study.

The segmentation ratio at the column outlet was set at 50% through the column centre [1]. Hence, 50% of the mobile phase exited the column through the peripheral port on the RF fitting: for further details on RF columns see reference [1].

Once the detection conditions were optimised, all subsequent analyses were conducted using gradient elution, specifically, a linear gradient was used, starting from initial conditions where the mobile phase was 5% A and 95% B; where A refers to 100% water at pH 9 buffered with ammonium acetate (0.1 M) and B was 100% methanol. The final mobile phase composition was 100% B obtained in 38 min. The mobile phase was held at 100% B for 10 min, and returned to the initial conditions in 2 min after which the column was equilibrated with 5-column volumes of mobile phase. The HPLC flow rate was 1.0 mL/min. The segmentation ratio at the column outlet was set at 50% through the column centre [1]. All injection volumes were 20 μL, performed in duplicate under ambient temperature.

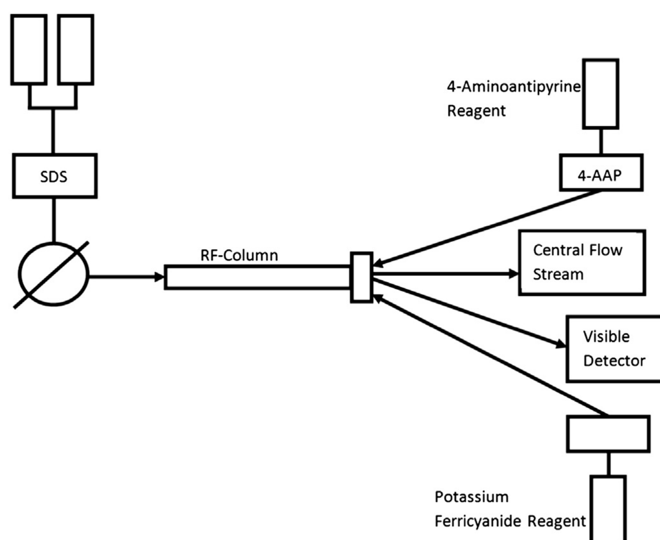

Fig. 1. Instrumental configuration for a system employing a reaction flow HPLC column with post column derivatisation. Components: RF-column = reactive flow column and 4-APP = 4-aminoantipyrine.

The instrumental setup for the two-component post-column derivatisation process is shown in Fig. 1. The reaction flow column has four ports: two peripheral ports were used to feed the post-column derivatisation reagents into the mobile phase flow, while one peripheral port was used to direct sample (derivatised or not) to a visible detector. The central flow port was directed to a collection vessel, although it is feasible to direct this sample to a separate detector and employ multiplexed detection in the assay [1,5]. This was not undertaken in the current study.

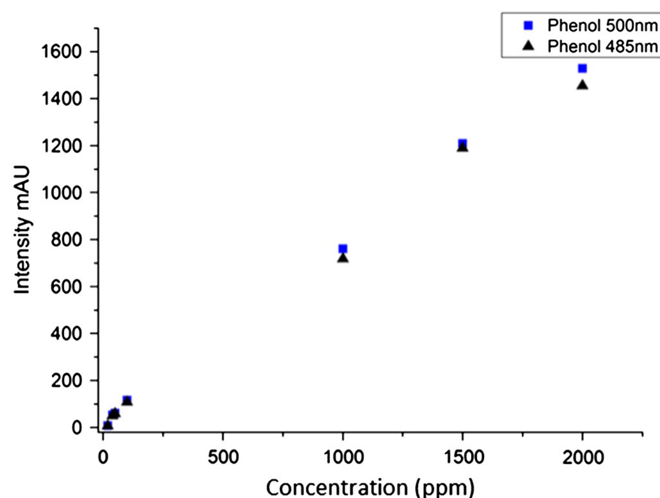

Fig. 2. Calibration curves for phenol. The blue data points represent the colorimetric response at 500 nm. The black data points represent the colorimetric response at 485 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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