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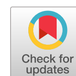
Bioactive screening of complex tea samples using the ferric reducing antioxidant power assay incorporating reaction flow HPLC columns for post column derivatisations



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ABSTRACT

This is the first study to employ a reaction flow (RF) HPLC column of a short 5 cm length to identify antioxidants in a variety of complex samples represented by tea, utilising simultaneous multiplexed detection techniques. The detection responses included: underivatised UV at 280 nm, underivatised fluorescence detection (FLD) and a post column derivatisation (PCD) colorimetric response for antioxidants via the ferric reducing antioxidant power (FRAP) assay. Both similarities and differences in the chromatograms obtained using the three detection modes highlight the power of multiplexed detection. The main findings from the bioactive profiling include: the Fruit of the Forest tea contains the lowest level of antioxidants and is clearly distinct from the other three teas which are based on *Camellia sinensis*. The antioxidants detected in the green tea and the two black teas are similar. The green tea contains a higher concentration of the two main antioxidants that eluted at 4.0 and 5.7 min. The simplicity of sample analysis and chemical profiling using RF columns and selective detection was the focus of this study via the analysis of antioxidants in tea using the FRAP reagent. The main advantage of RF-PCD is the ability to split flows for simultaneous detection with minimal post column dead volume contributions, making peak matching between detectors much easier. To date, few studies exploit the power of RF-PCD multiplexed detection to differentiate and identify bioactive compounds in various complex samples. Applications of the approach developed in this study may include antioxidant screening and profiling of natural products, foods and beverages.

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1. Introduction

In a recent communication [1] we described how reaction flow (RF) columns could be utilised for the analysis of antioxidants using the ferric reducing antioxidant power (FRAP) assay, which relies on the ability of antioxidants to reduce iron (III) to iron (II). This was the first study of its kind to employ RF columns for such assays, and only the second where the FRAP assay has been utilised in a post column derivatisation mode. In the analysis of antioxidants using the FRAP assay, the reduction of Fe(III) to Fe(II) is monitored by its interaction with 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), which forms a violet blue colour that can be detected at 593 nm [2–9]. Effectively, the FRAP assay is useful for any compound that exhibits a standard reduction potential (E^0) below +0.77 V [3], which is the case for a wide range of antioxidants, albeit, the ability to achieve detection is in part also dictated by the reaction rate [1].

Our prior study focused on optimisation of the FRAP assay in concert with a 150 mm RF column. We compared the RF mode of operation where no reaction loop is included to that of a variety of conventional modes of post column derivatisations that required reaction loops for successful antioxidant detection. The RF mode of operation was shown to be superior in performance compared to conventional modes of PCD, where the S/N ratio of the assay in RF mode was greater than the conventional mode, even when the conventional mode utilised reaction loops as large as 500 µL so as to improve reactivity in order to gain signal strength. The drawback of having such a large reaction loop was of course a substantial decrease in the separation efficiency. Furthermore, the RF-PCD method gave a wider linear range compared to the conventional technique with lower limits of detection and quantitation, better precision and greater linearity. Irrespective of whether or

Abbreviations: FRAP, ferric reducing antioxidant power assay; PCD, post column derivatisation; RF, reaction flow; AFT, active flow technology.

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not RF columns were employed, or conventional columns, the response factors varied from antioxidant to antioxidant, and a number of antioxidants that responded in the bench assay did not react to the FRAP reagent in PCD mode. Hence, at this point in time, very little is known regarding the usefulness of the FRAP assay in conjunction with RF columns for the analysis of complex samples. As such, this study serves to provide insight as to the applicability of PCD that utilises the FRAP in RF mode. We present here an analysis of a variety of tea samples undertaken using RF columns, with detection for the antioxidants at 593 nm, as well as simultaneous detection using a fluorescence detector (FLD).

2. Experimental

2.1. Chemicals

Mobile phase solvents were all of HPLC grade. Methanol was purchased from Thermo Fisher Scientific (Scoresby, Victoria, Australia) and Ultrapure Milli-Q water (18.2 MΩ cm) was prepared in-house and filtered through a 0.22 μm filter. Sodium acetate trihydrate, glacial acetic acid, hydrochloric acid 37%, 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) and ferric chloride hexahydrate were purchased from Sigma Aldrich (Castle Hill, New South Wales, Australia). All materials were used as received.

2.2. Reagents and sample preparation

The FRAP reagent was prepared according to the method outlined by Benzie and Strain [2]. Acetic acid buffer (300 mM, pH 3.6) was prepared by dissolving 40.8 g of sodium acetate trihydrate in 500 mL of Milli-Q water with the aid of ultrasonic agitation. The pH of the solution was then adjusted to 3.6 (±0.1) with glacial acetic acid and diluted to 1 L with Milli-Q water. HCl (40 mM) was prepared by diluting 3.3 mL of concentrated hydrochloric acid to 1 L with Milli-Q water. TPTZ (10 mM) was prepared by dispersing 62.5 mg TPTZ in 20 mL of 40 mM HCl with the aid of ultrasonic agitation. Ferric chloride (20 mM) was prepared by dissolving 108.1 mg ferric chloride hexahydrate in 20 mL of Milli-Q water with the aid of ultrasonic agitation. The final FRAP reagent was prepared by combining 500 mL of 300 mM acetic acid buffer pH 3.6, 20 mL of 10 mM TPTZ and 20 mL of 20 mM ferric chloride. The derivatisation reagent FRAP, was prepared daily and filtered through a 0.22 μm filter before use.

Four samples of different types of teas were used namely three from *Camellia sinensis* (English Breakfast (extra strong) tea, Earl Grey tea and Pure Green tea) from Twinings of London, and a herbal tea (Fruit of the Forest tea) from Tetley.

Tea samples were purchased from the local market (individual serving sachets/bags). Each tea sample for analysis was prepared by adding one tea bag to 40 mL of 80 °C tap water (with occasional agitation). Tap water was used without further treatment. After 10 min the bag was removed and the solution was allowed to cool to room temperature and an aliquot was filtered through a 0.22 μm PVDF syringe filter and used for analysis.

2.3. Instrumentation

All chromatographic experiments were conducted using an Agilent 1290 Infinity I system equipped with an Agilent 1290 auto-sampler, an Agilent 1290 binary pump and two modules of detection: Agilent 1260 DAD (1 μL flow-cell) and an Agilent 1260 FLD (8 μL flow-cell). One Shimadzu LC10ADvp pump, fitted with inline degassing unit (Phenomenex DG-4400 (Lane Cove, NSW, Australia)) was used to deliver the post column derivatisation reagent.

2.4. Chromatography column

Hypersil GOLD column (50 × 4.6 mm, particle diameter 3 μm) was used for reaction flow analysis and supplied by ThermoFisher Scientific (Runcorn, Cheshire, United Kingdom).

2.5. Chromatographic conditions

Analyses were performed under reversed phase gradient conditions at ambient temperature. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol. The linear gradient elution conditions from 0 to 30 min started at 5% B and ended at 100% B. The final composition was and held for an additional 4 min, returned to initial conditions in 0.1 min and re-equilibrated for 3 min. The column flow rate was set at 1.0 mL/min.

The segmentation ratio between the central and peripheral ports of the reaction flow column directed towards detection modules was 50:50. The instrumental set-up for the FRAP post-column derivatisation process is shown in Fig. 1. The FRAP reagent was delivered to one of the peripheral ports at the multiport outlet of the RF column at a constant flow rate of 0.5 mL/min. A second peripheral port allowed the derivatised eluent to exit the RF end fitting where it was passed to the DAD detector and the signal response was collected at 593 nm. The third peripheral outlet was blocked. The central flow port was directed to an FLD with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. Hence two modes of detection were employed simultaneously – antioxidant detection using PCD at 593 nm (visible) and FLD for underivatised compounds.

In addition, the samples were analysed a second time with the PCD reagent pump turned off, and the underivatised eluent flowing from the peripheral port was directed to the UV detector and analysed at a wavelength of 280 nm. Effectively this gave sample information across three modes of detection in two different sample injections.

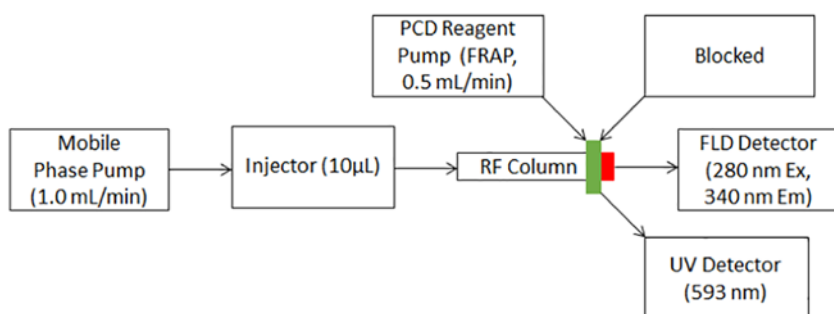

Fig. 1. Illustration of the instrumental setup for the derivatisation of antioxidants using the FRAP reagent and a reaction flow column with multiplexed detection of the underivatised central flow stream using a fluorescence detector. The green section of the RF column represents the radial outer peripheral section of the column that is separated from the red section representing the radial central portion of the column. (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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