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Total Antioxidant Capacity with Peak Specificity via Reaction Flow Chromatography and the Ferric Reducing Antioxidant Power Assay

Andrew Jones[1] · Agustín Acquaviva[2] · Thirada Suktham[1] · Gary R. Dennis[1] · R. Andrew Shalliker[1] · Arianne Soliven[1,3]

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Abstract

An established ferric reducing antioxidant power (FRAP) assay was optimised by preparation of the derivatisation reagent in 300 mM formate instead of 300 mM acetate conditions, resulting in increased sensitivity signal to noise responses by up to five to ten times. The quantitative protocol for selective detection of antioxidants via a HPLC post column derivatisation (PCD) technique using the 300 mM formate FRAP reagent conditions was then transformed into a high-speed qualitative screening protocol by utilizing an emerging technology 'reaction flow (RF) chromatography'. Reaction flow chromatography's ability to screen for total antioxidant capacity with additional peak specificity/profile information of active peaks could be achieved in under 2 min.

Keywords Total antioxidant capacity · Post column derivatisation · Reaction flow chromatography · Selective detection · Ferric reducing antioxidant power

Introduction

Total antioxidant capacity (TAC) measurements generally involve the manual mixing and derivatisation of processed and unprocessed food samples followed by UV–Vis spectroscopy detection and provide no peak specificity/profile of active peak(s) information (Szydłowska-Czerniak et al. 2012; Cömert and Gökmen 2018; Cai et al. 2019). Additionally, TAC assays are useful to eliminate antioxidant-poor and indicate antioxidant-rich sample candidates for high-resolution characterisation in complex samples encountered in early discovery research of new and/or alternative bioactive compounds in natural products (Wang et al. 2018; Sridhar and Linton 2019), and for the search of alternative additives for food and consumer products.

A number of antioxidant benchtop assay methods have been converted into online HPLC selective detection analyses via post-column derivatisation (PCD) (Zacharis and Tzanavaras 2013). For example, an antioxidant assay that incorporated the robustness and reproducibility of a HPLC separation coupled with chemiluminescence (CL) detection (Francis et al. 2010; McDermott et al. 2011). Alternative PCD techniques may have advantages or disadvantages in terms of detection sensitivity, specificity and selectivity for certain antioxidants (Acquaviva et al. 2018b); to date, no universal approach to rapidly screen for antioxidants exists. Furthermore, HPLC-PCD fails to serve as high-speed protocols due to the extra-column volume of the reaction coil. The additional post-column dead volume is required to enable sufficient mixing and reaction between the derivatisation reagent and the mobile phase exiting the column that contains the sample's eluting peaks (Zacharis and Tzanavaras 2013; Jones et al. 2015). The reaction coil's extra-column volume can be as large as 500 μL in a knitted coil fashion, which has a detrimental contribution to peak broadening and would obliterate the separation performance of small LC column formats typically employed for high throughput assays (Jones et al.

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✉ Arianne Soliven
a.soliven@westernsydney.edu.au; asoliven@fq.edu.uy

[1] Australian Centre for Research on Separation Science (ACROSS), School of Science and Health, Western Sydney University, South Parramatta Campus, Sydney, NSW 2150, Australia

[2] LIDMA (Laboratorio de Investigación y Desarrollo de Métodos Analíticos), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 and 115 (1900), La Plata, Argentina

[3] Department of Natural Products and Pharmacognosy, Faculty of Chemistry, Universidad de la República, General Flores 2124, 11800 Montevideo, Uruguay

2015). Despite these limitations, however, researchers still employ PCD techniques and large post-column reaction loops due to the valuable information that they provide; the importance of these techniques recently affirmed (Granato et al. 2018; de Camargo et al. 2019).

The development of post-column derivatisation without reaction loops utilise a new chromatography column technology – that is, a reaction flow chromatography (RF) column. These columns are in essence conventional HPLC columns, with a specially designed end fitting to facilitate mixing of the derivatisation reagents and eluting sample peaks in the column's outlet frit, a highly efficient mixing process that eliminated the peak band broadening contributions of the reaction loop and facilitates small column format coupling for high-speed analyses (Acquaviva et al. 2018a; Jones et al. 2018).

Recently, we have investigated the use of RF columns to perform high throughput analysis of phenolic compounds (Jones et al. 2018). The transfer of conventional reaction loop HPLC-PCD assays to RF-PCD assays and high-speed RF-PCD assays is not as easy as it seems, and in order to facilitate antioxidant screening, this study demonstrates the necessary hurdles to overcome and develop a high throughput RF-PCD antioxidant assay. We have selected green tea as a representative sample rich in antioxidants within a complex matrix; consumed globally as tea or added to other manufactured foods. This approach is not intended to substitute for higher resolution characterisation, but as an alternative approach to TAC measurements, initial assessments of the sample with additional peak specificity information typically employed as the initial screening of antioxidants in food science and natural product research.

Experimental

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 (Phenomenex, Lane Cove, NSW, Australia). Sodium acetate trihydrate, glacial acetic acid, ammonium formate, formic acid 100%, hydrochloric acid 37%, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate, ascorbic acid, gallic acid, (+)-catechin hydrate, chlorogenic acid, rutin hydrate, rosmarinic acid, morin, quercetin and thiourea were purchased from Sigma Aldrich (Castle Hill, New South Wales, Australia). All materials were used as received.

Reagents and Sample Preparation

The FRAP reagent was prepared according to the method outlined by Benzie and Strain (Benzie and Strain 1996). Acetate 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 the resultant solution was 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 acetate buffer pH 3.6, 20 mL of 10 mM TPTZ and 20 mL of 20 mM ferric chloride. The derivatisation reagent was prepared daily and filtered through a 0.45 μm nylon filter (Phenomenex, Lane Cove, NSW, Australia) before use.

Additionally, a modified FRAP reagent was prepared in 30 mM acetate buffer. The 30 mM acetate buffer was prepared by 1:10 dilution of the 300 mM acetate buffer with water. Forty mM HCl, 10 mM TPTZ and 20 mM $FeCl_3$ were prepared as per the FRAP reagent prepared in 300 mM acetate buffer. The final FRAP reagent was prepared by combining 500 mL of 30 mM acetate buffer pH 3.6, 20 mL of 10 mM TPTZ and 20 mL of 20 mM ferric chloride. The derivatisation reagent was prepared daily and filtered through a 0.45 μm nylon filter (Phenomenex, Lane Cove, NSW, Australia) before use.

Finally, a modified FRAP reagent was prepared in 300 mM ammonium formate buffer (pH 3.6). Formate buffer (300 mM, pH 3.6) was prepared by dissolving 18.9 g of ammonium formate 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 formic acid and the resultant solution was diluted to 1 L with Milli-Q water. 40 mM HCl, 10 mM TPTZ and 20 mM $FeCl_3$ were prepared as per the FRAP reagent prepared in 300 mM formate buffer. The final FRAP reagent was prepared by combining 500 mL of 300 mM formate buffer pH 3.6, 20 mL of 10 mM TPTZ and 20 mL of 20 mM ferric chloride. The derivatisation reagent was prepared daily and filtered through a 0.45 μm nylon filter (Phenomenex, Lane Cove, NSW, Australia) before use. Unless otherwise stated, all experiments were performed using the modified FRAP reagent prepared using formate buffer.

Two standard solutions were prepared, both at a concentration of 200 mg/L. Standard mixture 1 contained ascorbic acid, gallic acid, (+)-catechin hydrate and chlorogenic acid. Standard mixture 1 was prepared in 10:90 methanol:Milli-Q water. Standard mixture 2 contained rutin hydrate, rosmarinic acid, morin and quercetin. Standard mixture 2 was prepared in 40:60 methanol:Milli-Q water. Additionally, separate individual standards containing 200 mg/L ascorbic acid and 200 mg/L trolox were prepared in 10:90 methanol:Milli-Q water. The

standards were prepared by weighing 20 mg of each compound into a 100 mL volumetric flask and dissolved in the required volume of methanol (40 mL for standard 2, 10 mL for all other standard solutions) with the aid of ultrasonic agitation. The solutions were then cooled to room temperature and diluted to volume using Milli-Q water.

Pure Green tea from Twinings of London was purchased from the local market as individual serving sachets/bags and used to demonstrate the applicability of the method to a complex sample. The tea sample for analysis was prepared by adding one tea bag to 40 mL of 80 °C filtered tap water (with occasional agitation). 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 (Phenomenex, Lane Cove, NSW, Australia) syringe filter and used for analysis.

Instrumentation

All chromatographic experiments were conducted using an Agilent (Forest Hill, Victoria, Australia) 1290 Infinity I system equipped with an Agilent 1290 auto-sampler, an Agilent 1290 binary pump and an Agilent 1260 DAD (1 μL flow-cell). One Shimadzu LC10AD*vp* pump, fitted with inline degassing unit (Phenomenex DG-4400 (Lane Cove, NSW, Australia)), was used to deliver the post-column derivatisation reagent.

Chromatography Columns

A Hypersil GOLD column (30 × 4.6 mm, particle diameter 3 μm) fitted with a 4-port end fitting and a RF 2:1 frit supplied by Thermo Fisher Scientific (Runcorn, Cheshire, United Kingdom) was used. An unmodified Hypersil GOLD column of the same dimensions was used for conventional PCD analyses.

Chromatographic Conditions

The injection volume for all analyses was 5 μL. All analyses were performed using duplicate injections, unless stated otherwise. Analyses were performed under reversed phase gradient conditions at ambient temperature. Mobile phase A was 0.01 M hydrochloric acid in water and mobile phase B was 100% methanol. Standard mixture 1 and the individual ascorbic acid and thiourea solutions were analysed isocratically at a ratio of 90:10 (A:B). Standard mixture 2 was analysed isocratically at a ratio of 60:40 (A:B).

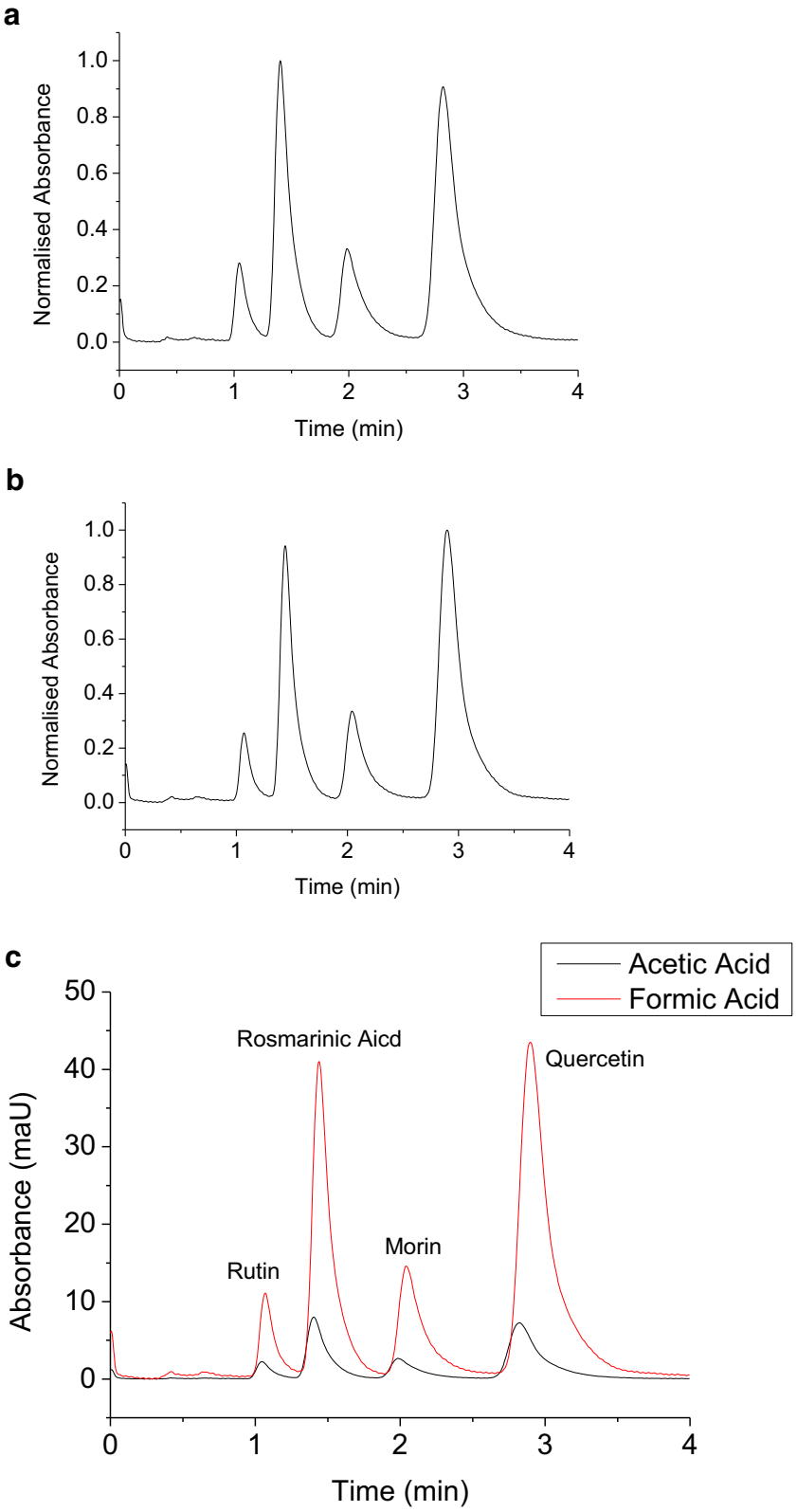
The conventional PCD approach is illustrated in Supplementary Fig. SF1 (top insert); the FRAP reagent was pumped at a flow rate of 0.5 mL/min into a zero dead volume T-piece where it was combined with the column effluent and then passed through a reaction loop (2, 20, 50, 100 or 500 μL), before it was passed to the detector or sent directly to the detector. All reaction loops were prepared using 0.254 mm i.d. PEEK tubing.

The instrumental set-up for the reaction flow FRAP RF process is shown in Supplementary Fig. SF1 (bottom insert). Supplementary Fig. SF2 illustrates the RF's specially designed end fitting: the top insert gives an expanded view of the three piece frit design, and the bottom insert showing the flow path directions of the four port outlet. 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 tubing connecting the RF column outlet to the detector inlet was made as short as possible, and the total extra-column/dead volume represented between the column outlet and detector (inclusive of the flow cell volume) was less than 2 μL, typical of the column to detector dead volume in modern HPLC systems without derivatisation techniques. The third peripheral outlet was blocked. The radial central flow port was directed to waste; it is feasible for the effluent from the radial central port to be directed to a second detector for analysis of the underivatised flow stream; however, this was not performed in this study. The ratio between the central (C) and peripheral (P) flows was set between 50:50 and 80:20 (C:P) (derivatisation pump turned off) by varying the length of tubing on the central port of the RF column. For the analysis of the tea samples, 40% of the mobile phase flowed through the peripheral port of the RF column, meaning that 60% of the mobile phase flowed through the central port.

The green tea sample was analysed at flow rates ranging from 1.0 to 5.0 mL/min using gradient conditions. Mobile phase A was 0.01 M hydrochloric acid in water and mobile phase B was 100% methanol. Initial conditions were 95:5 (mobile phase A: mobile phase B). A linear gradient of 3%/mL was used up to a final condition of 100% mobile phase B. The mobile phase was then returned to initial conditions and allowed to re-equilibrate with 5 column volumes prior to the next injection.

Results and Discussion

The majority of FRAP analyses employed via well plate readers/UV–Vis benchtop detectors to date have used 300 mM acetate buffer to prepare the FRAP derivatisation reagent originally established by Benzie and Stein in 1996 (Benzie and Strain 1996; Szydłowska-Czerniak et al. 2012). TAC often requires manual mixing of the sample and derivatisation reagent and does not provide any information about the main/minor active peaks that contribute to the TAC measurement. Subsequently, most FRAP HPLC-PCD assays


a
Normalised Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
Time (min)
b
Normalised Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
Time (min)
c
Acetic Acid
Formic Acid
Rosmarinic Aicd
Quercetin
Morin
Rutin
Absorbance (maU)
50
40
30
20
10
0
0
1
2
3
4
Time (min)

◀ **Fig. 1** Effect of FRAP reaction under different buffer conditions for conventional PCD, 2 μL reaction loop, chromatograms of standard separations: **A** 300 mM acetate, **B** 30 mM acetate and **C** 300 mM formate (overlaid with **B** for comparison)

also use the 300 mM acetate buffer (Raudonis et al. 2012; Jones et al. 2017; Raudone et al. 2017).

The use of a formate buffer over an acetate buffer increased the reactivity of the FRAP reagent leading to greater signal to noise (S/N); required for higher throughput PCD analyses where residence time between mixing of the FRAP reagent with the column effluent is limited. When the FRAP 300 mM acetate derivatisation reagent was paired with HPLC-PCD low volume reaction coils (required for high-speed screening assays) the 300 mM acetate conditions resulted in significant peak tailing (Fig. 1a). Figure 1b shows a chromatogram of the same standards analysed when using FRAP prepared in 30 mM acetate buffer using the same 2 μL loop. The peak tailing is improved (although not entirely removed) by lowering the acetate concentration. A series of benchtop tests were conducted in order to eliminate the acetate buffer from the FRAP reagent. It was established that buffering was required in order to maintain reaction speed. A number of common buffers were considered including phosphate and citrate; however, these were not suitable due to insolubility of their iron (II) and/or iron (III) salts.

Iron (II) and Iron (III) formate salts are known to be soluble and formate buffers at an appropriate pH for the FRAP reagent. The use of a 300 mM formate buffer was found to improve the analysis, although it appears that the tailing is marginally decreased (Fig. 1c). The formate buffer increased S/N by five to ten times compared to the acetate buffer, as shown in Fig. 1c. Lower concentrations of formate were trialled without significant gains in either signal response or peak shape. Hence, 300 mM formate was the final conditions of the FRAP reagent buffer.

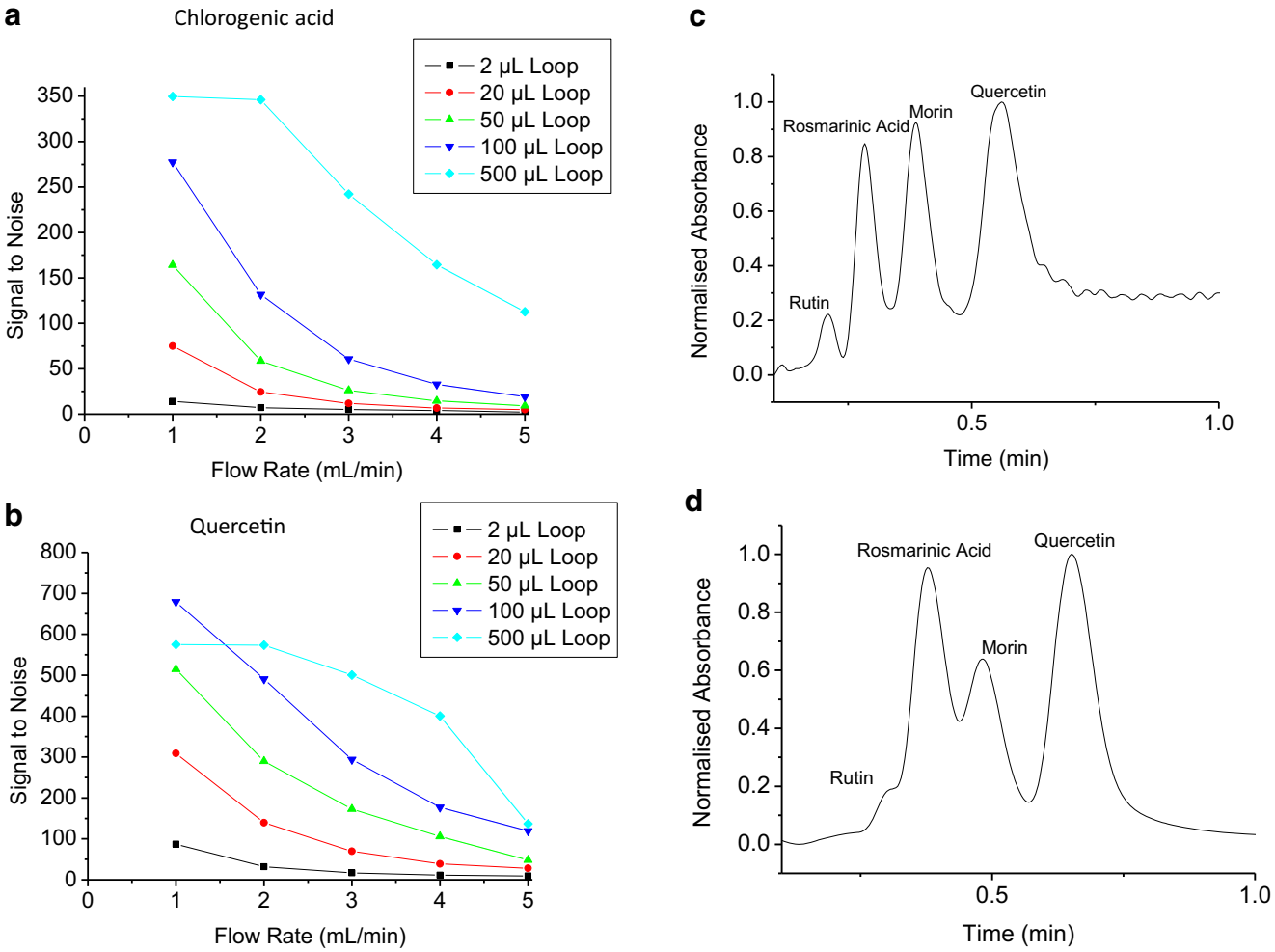


Fig. 2 Effect of reaction loop volume (2, 20, 50, 100 and 500 μL) and flow rate (1.0 to 5.0 mL/min) on the S/N for conventional PCD: **A** chlorogenic acid and **B** quercetin. Chromatograms of standard separations at 5 mL/min for the **C** 2 and **D** 500 μL loop

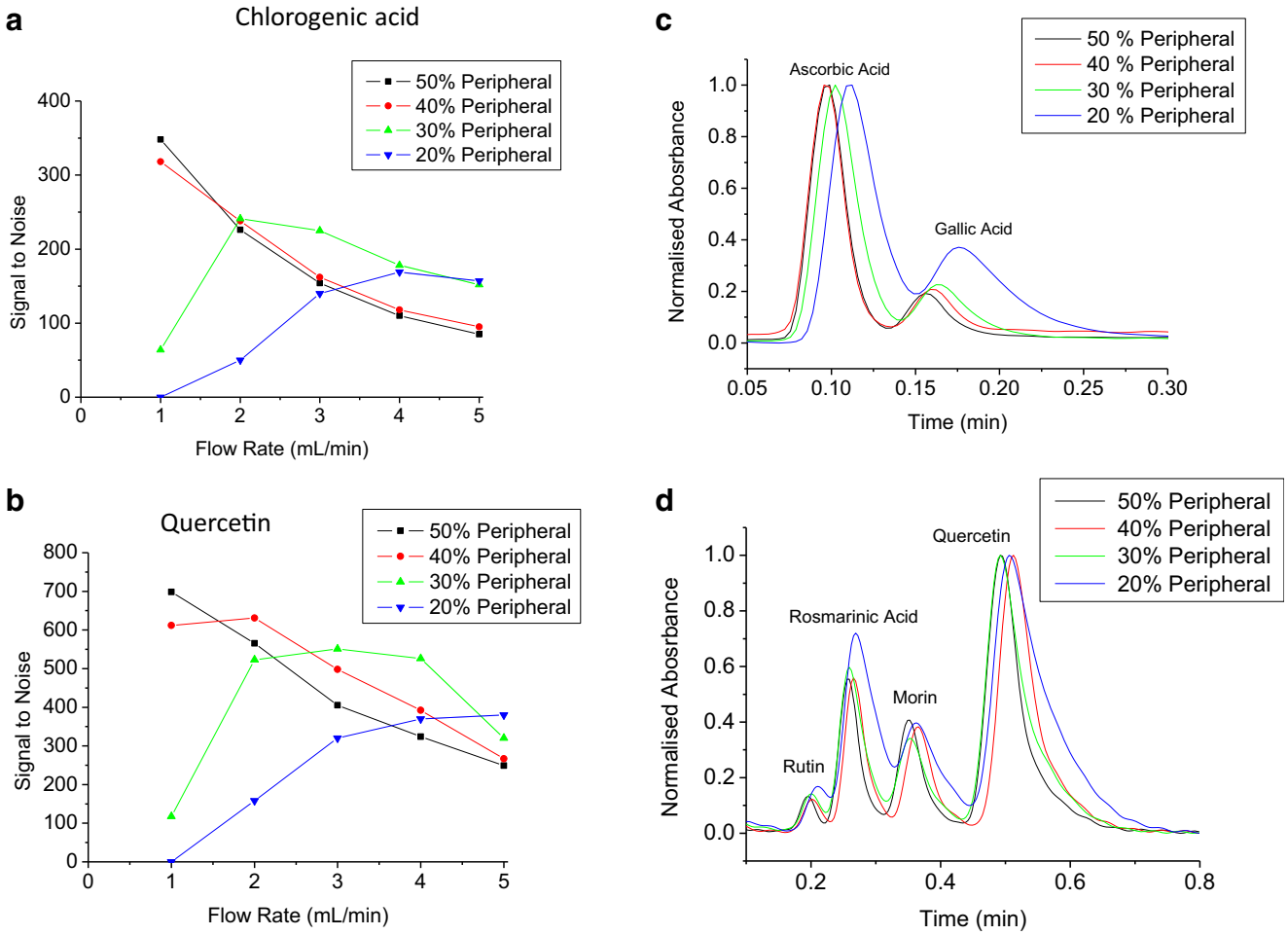


Fig. 3 RF operated at different peripheral segmentation ratios. S/N ratios for **A** chlorogenic acid and **B** quercetin at flow rates 1.0 to 5.0 mL/min. Chromatograms of **C** ascorbic acid and gallic acid and **D** rutin, rosemaric acid, morin, quercetin fixed at 5.0 mL/min at different segmentation ratios of the RF column

In order to develop the qualitative HTP screening approach using the optimized 300 mM formate FRAP conditions, a quantitative study was established, and the calibration results for a set of 16 standards are listed in Supplementary Information Table 1, and each antioxidant's relative response factors to TROLOX are listed in Supplementary Information Table 2 (Suktham et al. 2019). This method was then transformed into a TAC HTP qualitative assay by utilizing a shorter 3 cm column length with an approximate column volume of 350 μL in order to reach a higher maximum allowable column flow rate with reduced backpressure and minimize the total analysis time. Fundamentally, HTP PCD antioxidant assays are difficult to couple with PCD due to the fact that the column volume is greatly exceeded by the 500 μL reaction loop volume required for detection sensitivity and on the other hand sacrifices the separation resolution due to its extra column dispersion contributions (Raudonis et al. 2012; Zacharis and Tzanavaras 2013; Jones et al. 2015; Arslan Burnaz et al. 2017; Raudone et al. 2017).

To illustrate this fact, analyses using the conventional 3 cm HPLC column PCD approach in a high throughput mode of operation was performed for standard separations, at flow rates: 1.0, 2.0, 3.0, 4.0 and 5.0 mL/min and reaction loops: 2, 20, 50, 100 and 500 μL. Figure 2 shows the S/N obtained using chlorogenic acid (Fig. 2a) and quercetin (Figure 2b), respectively. Generally, S/N decreased with faster velocities, and larger reaction loop volumes improved the S/N. Figures 2c and 2d illustrated that at the highest velocity (5 mL/min), the detrimental loss in separation resolution when the reaction loop was increased from 2 to 500 μL.

Reaction flow (RF) chromatography is an emerging technology that eliminates the HPLC-PCD post-column dispersion and volume associated with the reaction loop, as the PCD reaction occurs within the column outlet (Supplementary Fig. SF2). Therefore, RF enables the coupling of PCD with fast velocities/reduced analysis time, required for the development of an automated total antioxidant capacity measurement with peak specificity.

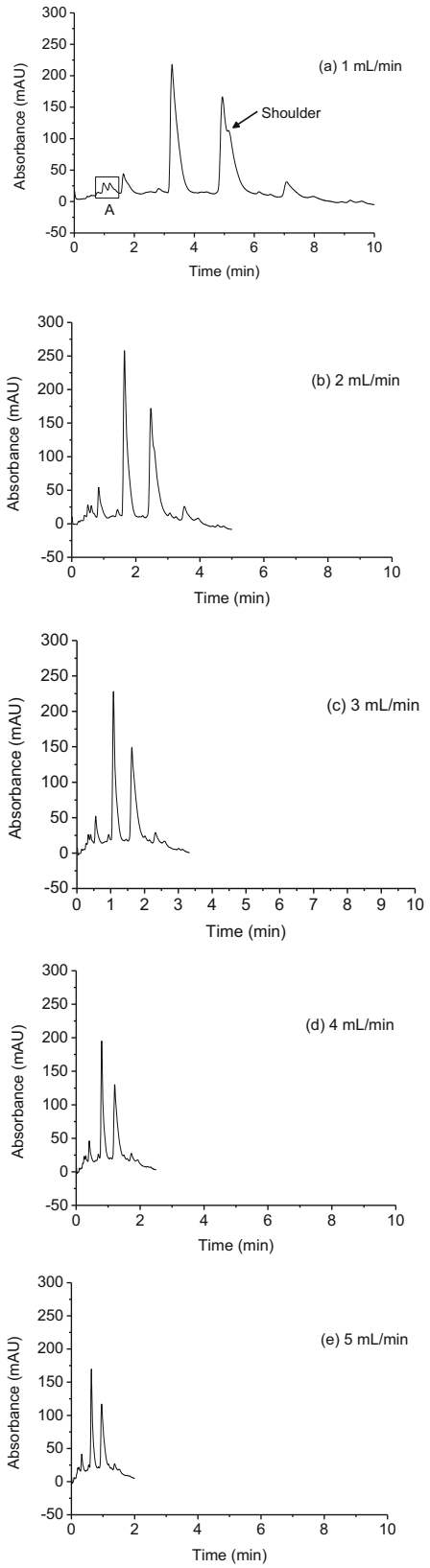


Fig. 4 RF total antioxidant screening with peak specificity of green tea at flow rates: **A** 1.0, **B** 2.0, **C** 3.0, **D** 4.0 and **E** 5.0 mL/min

The S/N responses for different RF peripheral port segmentation ratios (20% to 50%) and increased column velocities were tested, and the chlorogenic acid and quercetin plotted in Figs. 3a and 3b. The 40 and 50% segmentation ratios (that is, 0.4 and 0.5 mL/min passing through the peripheral port and being derivatised, respectively) S/N generally decreased as the flow rate increased. The lower segmentation ratios 20 and 30% resulted with the largest S/N at the highest flow rate at 5 mL/min. This may be associated with the lower volumetric flow rate of 0.2 and 0.3 mL/min, respectively, that enabled increased residence time for the reaction to occur in the outlet and detector flow cell with respect to the higher segmentation ratios/volumetric flow rates. At the highest velocity (5 mL/min), the lowest segmentation ratio suffered from the largest peak widths compared to the other higher segmentation ratios (Figs. 3c and 3d). Hence, a compromise between peak shape and S/N must be tuned for each particular separation problem.

To demonstrate the applicability of the RF-PCD FRAP assay as an automated TAC assay with peak specificity, a complex natural product represented by green tea was selected for its antioxidant activity, complex matrix and previous use to demonstrate selective detection with high throughput phenolic capacity measurements (Acquaviva et al. 2018a; Jones et al. 2018). This screening technique may be extended to other complex matrices provided no precipitation occurs between the sample, mobile phase and derivatization reagent – detrimental to the detector's flow cell.

The chromatograms in Figs. 4a to 4e illustrate the antioxidant active peaks/profile that contributes to the total antioxidant response with increased velocities from 1.0 to 5.0 mL/min. The fixed RF segmentation ratio of 40% from the peripheral was an intermediate compromise between peak width and S/N. Figure 4a shows two main active peaks at retention times of 3.5 min and 5 min, respectively. Additionally, a shoulder on the peak eluting at 5 min was observed indicating that at least two chemical species were closely eluting under the conditions of the method. A number of minor antioxidant components were also observed in the chromatogram, including three peaks that eluted with retention times between 1.0 and 2.0 min (labelled as 'A' in Fig. 4a) and a peak that eluted with a retention time of 7 min.

As with the separations of the standard mixtures, it was expected that the resolution would decrease as the flow rate increased, and this was the case for the complex tea sample. This is a function of high throughput analysis rather than the RF process. This can be observed in the shoulder of the peak that corresponds to the peak eluting at 5 min in the chromatogram obtained using a flow rate of 1.0 mL/min. At flow rates of 3.0 mL/min and greater, the shoulder was no longer observed; instead, a peak that appeared to have greater tailing was observed than when the compounds were partially separated. Additionally, the resolution between the peaks labelled as 'A' in Fig. 4 decreased as the flow rate increased, although

these three peaks were all still visibly resolved even at 5.0 mL/min. Also, as expected, sensitivity decreased as the flow rates increased due to decreased reaction time in the column outlet.

The chromatographic data in Fig. 4d clearly shows that high-speed separations via RF PCD can yield very detailed initial screening information relating to antioxidant profiles of complex samples. For example, the 2-min analysis approach can rapidly screen the sample's total antioxidant capacity and provided a profile illustrating two main peaks and various minor actives, an informative qualitative report to guide further structure elucidation and quantitation, for example, via LC-MS/MS, compared to the 96 well plate reader and/or a benchtop UV-Vis spectrophotometer approaches that require time for manually mixing the reagent with the sample, prone to human error and do not provide peak specificity. Extending the speed and selectivity for other reaction schemes is out of the scope of this study. The HTP RF PCD protocol developed in this study may facilitate as an automated total antioxidant response assay with peak specificity. Potential applications of this rapid screening protocol would benefit food research laboratories searching for alternative antioxidant additives for consumer products.

Conclusions

A rapid antioxidant selective detection assay via reaction flow chromatography FRAP assay was optimized and developed in this study, an alternative measurement of total antioxidant capacity assay with peak specificity/activity profile, illustrated by the ability to screen a complex antioxidant rich sample under two minutes. Note, this methodology does not serve to substitute higher resolution selective detection techniques, but a qualitative screening protocol employed to automatically derivatise and screen for antioxidant capacity for numerous samples encountered in food research. Furthermore, this HTP TAC approach has the potential as an initial screening assay for the search of alternative antioxidants to be used as food additives. The cost/time benefits of transforming the quantitative protocol into a HTP TAC protocol incorporating a HPLC separation may increase the productivity of laboratories to screen and profile for antioxidant activity before higher resolution quantification.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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