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Highlights

- High throughput reaction flow chromatography total antioxidant capacity measurement
- Total analysis time of four minutes - automated mixing and antioxidant profile
- Bench top approaches do not yield an antioxidant profile and requires manual mixing
- Potential uses: antioxidant screening, stability and/or adulteration testing

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Better than bench top. High speed antioxidant screening via the cupric reducing antioxidant capacity reagent and reaction flow chromatography

Thirada Suktham[a], Andrew Jones[a], Agustín Acquaviva[b], Gary R. Dennis[a], R. Andrew Shalliker[a], Arianne Soliven[a,c*]

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

[b] *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.*

[c] *Grupo de Análisis de Contaminantes Traza, Cátedra de Farmacognosia y Productos Naturales, Facultad de Química, Universidad de la República, General Flores 2124, 11800 Montevideo, Uruguay.*

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Keywords: total antioxidant capacity; reaction flow chromatography; post column derivatisation; selective detection; cupric reducing antioxidant capacity

Corresponding author details: a.soliven@westernsydney.edu.au / asoliven@fq.edu.uy

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Abstract

This study is based upon a recently established method for quantification of the antioxidant capacity of natural samples via a HPLC separation and a hyphenated selective detection (post-column derivatization with cupric reducing antioxidant capacity reagent) technique. This protocol demonstrated the main improvements to transform the quantitative protocol into a high-speed qualitative automated assay to screen samples for their potential total antioxidant capacity, typically performed via manual mixing of the sample and derivatisation and measured on a 96 well plate reader/bench top UV-Vis spectrophotometer. This approach with automated mixing is a more informative alternative for total antioxidant capacity as the antioxidant peaks are profiled for each sample within four minutes. This antioxidant profile may be used for routine analysis of raw materials and/or a guide for targeted approaches for structure elucidation for laboratories interested in early drug discovery, natural product research and the search of alternative antioxidant additives in consumer goods/therapeutics. This technique could also be used to monitor the stability, alteration or adulteration of manufactured goods containing antioxidants.

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1. Introduction

High throughput (HTP) antioxidant screening approaches involve manual mixing of the sample and derivatisation reagents in a 96 well plate reader tray or a cuvette, followed by a measurement using a UV-Vis detector; they are advantageous as they provide fast measure of the total antioxidant activity (TAC) and may be easily applied in various food and natural product samples [1–3]. HTP TAC techniques are able to rapidly screen samples, subsequently filter or nominate only antioxidant rich samples for further high resolution characterization of the antioxidant activity via more time consuming LC-MS/MS, which is also more expensive to undertake (labour, expertise, consumables and waste generation) [4,5]. The disadvantages of screening via 96 well plate reader/bench top assays are associated to the manual mixing/handling of the sample mixing with the derivatisation reagent, the main bottle-neck of the workflow subject to a higher risk of human error.

An alternative approach for rapid screening of antioxidant activity via an "automated TAC measurement with peak specificity" approach utilised the ferric reducing antioxidant power (FRAP) assay and reaction flow (RF) chromatography [6]. The initial FRAP 300 mM acetate conditions via Benzie and Strain [7] is not only difficult to reach the required pH via titration with acid, but also resulted in lower S/N responses compared to the formate conditions [6]. The FRAP RF screening protocol involves a modernised HPLC approach, adopting the post-column derivatisation (PCD) assay with RF chromatography columns, where no reaction loop is required, and subsequently the separation and detection performance outperformed conventional approaches. The constituents of the sample are resolved from one another and derivatised within the column outlet before a detection response of the antioxidant activity is measured. Hence, the robustness and reproducibility of the FRAP RF workflow for the TAC measurements and HTP antioxidant profiling are tightly

coupled with the HPLC system's auto-sampler, RF column separation and gradient pumps to perform the separation and selective detection.

Recently, the quantitative RF FRAP assay was compared to the RF cupric reducing antioxidant capacity (CUPRAC) approach for quantitation of antioxidants [8]. The CUPRAC RF protocol was the methodology of choice as it provided a higher level of performance compared to the FRAP assay, demonstrating less baseline noise interference, greater sensitivity, wider linear dynamic range and better precision [8].

To date HTP qualitative selective detection RF protocols have been limited to two studies (i) phenolic profiling assay [9] and (ii) antioxidant profiling via the improved FRAP assay using 300 mM formate buffered derivatisation reagent conditions [6]. As it is a long term aim of our research to develop highly efficient selective detection assays that can exploit the workhorse of analytical laboratories – the HPLC-UV system, we present here a study on the use of the CUPRAC assay for the HTP analysis of antioxidants in standard materials and natural samples. The method presented is an alternative to the TAC 96 well plate reader or the bench top UV spectrophotometer approaches. This is only the third study to explore high speed RF selective detection processes and extends the work to include CUPRAC assays. As there is no universal approach for antioxidant capacity measurements [10], we expand the HTP RF rapid screening protocols to include antioxidant selective detection via a CUPRAC approach.

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2. Experimental

2.1 Chemicals

Ultrapure Milli-Q water (18.2 MΩ cm) was prepared in-house and filtered through a 0.2 µm filter. All chemicals of analytical reagent grade were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) including methanol, formic acid, copper (II) chloride,

ammonium acetate, 2,9-dimethyl-1,10-phenanthroline (neocuproine). Also, chemicals from Sigma-Aldrich (Castle Hill, NSW, Australia) were used as antioxidant standards, namely, gallic acid, catechin, caffeic acid, rosmarinic acid, quercetin, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) and chrysin.

2.2 Preparation of samples and reagents

The CUPRAC reagent was prepared by combining solutions of 10 mM copper (II) chloride solution, 7.5 mM neocuproine solution and 1 M ammonium acetate buffer pH 7.0 at a ratio of 1:1:1 by volume [11]. 0.511 g of $CuCl_2.2H_2O$ in was dissolved in 300 mL of water and 0.469 g of neocuproine into 300 mL of methanol. Finally, 23.124 g of ammonium acetate was dissolved in in 300 mL of water and the pH was adjusted to 7.0 (±0.1). All solutions were then mixed thoroughly and filtered through a 0.45µm nylon filter prior to use.

The stock standard solution was prepared at a concentration of 1000 mg/L by dissolving 50 mg of each antioxidant in methanol and diluting quantitatively to 50 mL. The standard set consisted of gallic acid, catechin, caffeic acid, rosmarinic acid, quercetin, trolox and chrysin. The standard mixture solution at the concentration of 100 mg/L was prepared by dilution of the stock standard with methanol.

Pure Green, Earl Grey, Camomile and English Breakfast tea samples (as bags) were purchased from the local market. The tea samples for analysis were prepared by adding one tea bag to 40 mL of 80ºC filtered tap water and allowing the bag to rest for 10 minutes with occasional agitation. The bag was removed, an aliquot taken and filtered through a 0.45 µm nylon filter prior to analysis.

2.3 Instrumentation

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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 LC10ADvp pump, fitted with inline degassing unit (Phenomenex DG-4400 (Lane Cove, NSW, Australia)) was used to deliver the post-column derivatisation reagent.

A Hypersil GOLD C18 (100 × 4.6 mm, 3 µm D_p) column was used for the underivatised method; whereas, a Hypersil GOLD C18 (100 × 4.6 mm, 3 µm D_p) Active Flow Technology (AFT) column with a 2:1 frit and 4-port outlet head-fitting was used for the reaction flow PCD studies. The PCD reagent was delivered to one of the three-peripheral ports on the outlet end-fitting of the RF column. Subsequently the mobile phase, antioxidants and PCD reagents were mixed inside the RF end-fitting and exited via a second outlet peripheral port. The derivatised mixture was then delivered to the DAD detector. The remaining peripheral outlet was plugged. At the central port, underivatised sample eluted and this was either directed to waste or the DAD detector (as specified when relevant). A schematic diagram of the RF process can be found in Supplementary Figure 1.

2.4 Chromatographic separations

Separations were undertaken using gradient elution for both the standard mixes and the natural samples. Mobile phase A was 0.1 % formic acid in Milli-Q water and mobile phase B was 0.1 % formic acid in methanol. Prior to any injection the column was equilibrated with three column volumes of the initial mobile phase (95 %A/5 %B). At injection the mobile phase composition changed in a linear manner up to the composition 100 % B over a period of 19 minutes. The final composition was held for 1 minute. Then, mobile phase composition was returned to the initial conditions in a period of 0.1 minutes and re-equilibrated with at least 3 column volumes before the next analysis. The flowrate was 1.0

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mL/min, unless specified. Linear gradients at higher flowrates maintained a gradient rate of change of 5 %/mL. All injection volumes were 10 µL. The detector was set to a scan mode from 200 nm to 600 nm. The analysis wavelengths were set to 450 nm (2 nm bandpass) for CUPRAC, and 280 nm (2 nm bandpass) for underivatised analysis, respectively.

The segmentation ratio at the column outlet was initially set at 50 % through the column centre [8]. Hence, 50 % of the mobile phase exited the column through the peripheral port on the RF fitting. The PCD reagent was delivered to the peripheral port of the RF column at a flowrate of 0.5 mL/min.

3. Results and Discussion

This paper highlights the main practical transformations of the RF CUPRAC quantitative protocol where the flowrate is fixed at 1.0 mL/min and segmentation ratio set to 50% [8], into a HTP antioxidant screening protocol for complex samples. The original method's calibration results and equivalent response factor to trolox are listed in Supplementary Tables 1 and 2, respectively. The aim of this study was to provide an alternative methodology that can be implemented in labs that require initial HTP screening of a sample's total antioxidant capacity with an additional profile of only the active peaks. The noise was taken from three different sections of the separation, averaged, and then used to calculate the signal to noise (S/N) of each peak (the peak height taken as the signal). The elution order of the antioxidant peaks is as follows: gallic acid, catechin, caffeic acid, rosmarinic acid, quercetin, trolox and chrysin.

The optimisation of the RF chromatography segmentation/split ratio was focused on maximising the S/N with the RF column flowrate set at 1.0 mL/min, results shown in Figure 1(a). For all studied antioxidant standards, the S/N increased with the increased RF segmentation ratio, hence the RF column's outlet was then fixed at 60%. Due to the

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decreased residence time of the reaction/derivatization with increased flowrates, we limited the highest tested ratio to 60%.

Figure 1(b) shows that the S/N increased with faster flowrates/reduced analysis time when the segmentation ratio was fixed at 60%. The S/N values are also listed in Table 1 for closer inspection, the minor exception was for chrysin's S/N, which stayed constant when the flowrate was increased from 3.0 to 4.0 mL/min. The final approach with a segmentation ratio of 60% and flowrate of 4.0 mL/min is recommended as an RF qualitative HTP screening protocol, while we recommend the original method for quantitative purposes with the drawback of longer analysis time [8].

The peak widths, retention times, and normalised values (normalised by multiplication of the flowrate) are listed in Table 1. The normalised peak width SD were between 0.03 and 0.11 min and the normalised retention times % RSD values were between 0.48-1.61. Representative of both the LC system's reproducible gradient delivery and the precision of the column's retention behaviour at increased velocities/pressures. The peak widths decreased as flowrate/pressure increased, in agreement with operating at the highest allowable flowrate to maximise peak capacity for small gradient reversed phase separations [12].

Before showcasing the high throughput screening of various samples via RF CUPRAC, a comparison of the underivatised UV chemical profile of the complex sample represented by Green tea compared to the derivatized antioxidant profile is illustrated in Figure 2. The main advantage of RF chromatography even when performed at higher velocities is its ability to easily match peaks between the two profiles. At 4.0 mL/min with a segmentation ratio of 60% a simple visual inspection of the derivatised antioxidant profile and the non-derivatised UV profile can easily point out that the main component in Green tea was detected at 280 nm with a response of 1948 mAU, while the antioxidant profile

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illustrated that it had the second highest antioxidant activity. Most importantly, the largest antioxidant peak measured at 450 nm with a response of 429 mAU, had a non-derivatised UV response at 280 nm that indicated it was approximately 19 times less concentrated than the main component peak in Green tea as observed in the underivatised chromatogram. Hence, this highlights an alternative approach not only for TAC screening of samples, but together with the underivatised UV profiles (repeating the same injection with the derivatisation pump switched off), can be a useful strategy to find alternative/undiscovered antioxidant peaks in natural products.

Screening of different tea samples via the HTP CUPRAC RF protocol is illustrated in Figure 3, with the detection response fixed for comparative purposes between samples (a) Green, (b) Earl grey (c) Camomile and (d) English Breakfast teas. Camomile tea is a herbal tea while Green, Earl Grey and English Breakfast teas belong to the family *Camellia sinensis*. The advantage of this protocol is that the data analysis simply involves the visual inspection of the antioxidant profiles. While all profiles are unique, we have indicated in each antioxidant profile three sections of interest for peaks eluting between (a) 0.6-0.8 min, (b) 0.9-1.1 min and (c) 2.1-2.3 min. The herbal tea is quite distinct from the *Camellia sinensis* teas with a completely different profile. A closer inspection of the Camomile tea profile shows that it has the same antioxidant peaks with the same retention time (sections b and c), but at relatively much lower concentration compared to the *Camellia sinensis* teas. A comparison of the profiles of *Camellia sinensis* teas shows that a,b and c are different in their ratios, revealing potential markers for teas belonging to this same family. Green tea and Earl grey are quite different overall in their profiles but have comparable peak ratios for the section c of their profiles. English Breakfast tea is distinctly different compared to the Green and Earl grey *Camellia sinensis* teas as the later eluter in section c having been characterized with relatively higher antioxidant activity.

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If we were to blind the sample names, to mimic natural product/early drug discovery research programs for the search of alternative bioactives, the activity profiles of Green tea, Earl Grey and English Breakfast would indicate high antioxidant activity samples nominated for further characterisation, while Camomile represents low activity, a sample that may not necessarily need to be analysed further.

The potential impact of this methodology for producing HTP TAC profiles could be useful for end-users in routine QC/QA or R&D labs to easily compare samples to one another to determine antioxidants that are potentially unique or common to one another, with large activity for the search of unique alternative antioxidants for consumer product manufacturing [13], or for early drug discovery [14]. We limit these conclusions at this point in time to teas, which represent only a small fraction of antioxidant rich consumer products. Potentially the impact of this methodology and future studies could also be extended to the suitability of this protocol for routine testing/profiling of raw materials/natural products for the manufacturing of antioxidant consumer products and monitoring stability, adulteration or blending of antioxidants in final products.

4. Conclusion

This study outlined the development of a quantitative protocol recently developed by our group into a high throughput, better than the traditional bench top protocol approach to measure total antioxidant capacity with the advantage of providing an antioxidant profile of the sample's activity. The use of seven antioxidant standards demonstrated the highest S/N ratio was achieved when the RF column segmentation ratio was fixed at 60% via the peripheral outlet and flowrate set at 4.0 mL/min. The RF workflow reproducibility across different velocities from 1.0 to 4.0 mL/min were between 0.48 and 1.61 % RSD and peak width SD were between 0.03 and 0.11 min. This protocol has the potential impact to be used

to find alternative antioxidant peaks in natural products, which may not necessarily be the most concentrated constituent but possess stronger antioxidant activity. Also, this methodology demonstrated its ability to screen and nominate/prioritise antioxidant rich samples for further characterisation/structure elucidation. Potentially, it may be useful as an initial assessment for antioxidant profiling for stability, manufacturing and adulteration testing of antioxidant consumer products.

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5. References

[1] H. Cai, Q. Zhang, L. Shen, J. Luo, R. Zhu, J. Mao, M. Zhao, C. Cai, Phenolic profile and antioxidant activity of Chinese rice wine fermented with different rice materials and starters, Lwt. 111 (2019) 226–234. doi:10.1016/j.lwt.2019.05.003.

[2] E.D. Cömert, V. Gökmen, Evolution of food antioxidants as a core topic of food science for a century, Food Res. Int. 105 (2018) 76–93. doi:10.1016/j.foodres.2017.10.056.

[3] A. Szydłowska-Czerniak, A. Tułodziecka, E. Szłyk, Determination of Antioxidant Capacity of Unprocessed and Processed Food Products by Spectrophotometric Methods, Food Anal. Methods. 5 (2012) 807–813. doi:10.1007/s12161-011-9314-1.

[4] L. Wang, Y. Luo, Y. Wu, F. Xia, Z. Wu, Quickly verifying the antioxidant

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contribution of the individual composition in natural antioxidants by HPLC-free radical scavenging detection, Lwt. 96 (2018) 461–468. doi:10.1016/j.lwt.2018.06.005.

[5] K. Sridhar, A. Linton, In vitro antioxidant activity of Kyoho grape extracts in DPPH % and ABTS % assays : Estimation methods for EC 50 using advanced statistical programs, Food Chem. 275 (2019) 41–49. doi:10.1016/j.foodchem.2018.09.040.

[6] A. Jones, G.R. Dennis, R.A. Shalliker, A. Jones, Total Antioxidant Capacity with Peak Specificity via Reaction Flow Chromatography and the Ferric Reducing Antioxidant Power Assay Total Antioxidant Capacity with Peak Specificity via Reaction Flow Chromatography, Food Anal. Methods. Submitted (2019).

[7] I.F.F. Benzie, J.J. Strain, The Ferric Reducing Ability of Plasma (FRAP) as a Measure of “Antioxidant Power”: The FRAP Assay, Anal. Biochem. 239 (1996) 70–76. doi:10.1006/abio.1996.0292.

[8] T. Suktham, A. Jones, A. Soliven, G.R. Dennis, R.A. Shalliker, A comparison of the performance of the cupric reducing antioxidant potential assay and the ferric reducing antioxidant power assay for the analysis of antioxidants using reaction flow chromatography, Microchem. J. 149 (2019) 104046. doi:10.1016/j.microc.2019.104046.

[9] A. Jones, A. Acquaviva, G.R. Dennis, R.A. Shalliker, A. Soliven, High throughput screening of phenolic constituents in a complex sample matrix using post-column derivatisations employing reaction flow HPLC columns, Microchem. J. 141 (2018). doi:10.1016/j.microc.2018.03.026.

[10] M. Özyürek, K. Güçlü, R. Apak, The main and modified CUPRAC methods of antioxidant measurement, TrAC - Trends Anal. Chem. 30 (2011) 652–664. doi:10.1016/j.trac.2010.11.016.

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[11] S.E. Çelik, M. Özyürek, K. Güçlü, R. Apak, Determination of antioxidants by a novel on-line HPLC-cupric reducing antioxidant capacity (CUPRAC) assay with post-column detection, Anal. Chim. Acta. 674 (2010) 79–88. doi:10.1016/j.aca.2010.06.013.

[12] A. Soliven, I.A. Haidar Ahmad, M.R. Filgueira, P.W. Carr, Optimization of gradient reversed phase chromatographic peak capacity for low molecular weight solutes, J. Chromatogr. A. 1273 (2013) 57–65. doi:10.1016/j.chroma.2012.11.068.

[13] M. Carocho, P. Morales, I.C.F.R. Ferreira, Antioxidants: Reviewing the chemistry, food applications, legislation and role as preservatives, Trends Food Sci. Technol. (2018). doi:10.1016/j.tifs.2017.11.008.

[14] D.A. Dias, S. Urban, U. Roessner, A Historical Overview of Natural Products in Drug Discovery, Metabolites. 2 (2012) 303–336. doi:10.3390/metabo2020303.

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Figure Captions

Figure 1. S/N of seven standards (peaks numbered in elution order: 1 – gallic acid, 2 – catechin, 3 – caffeic acid, 4 – rosmarinic acid, 5 – quercetin, 6 – trolox, 7 – chrysin, same traces used in both inserts) under RF gradient separation conditions and CUPRAC selective detection at 450 nm with respect to (a) the segmentation ratio of the RF column, fixed flowrate at 1 mL/min; and (b) increased flowrate (mL/min), with the segmentation ratio fixed at 60%.

Figure 2. Green tea's UV/non-derivatized chemical profile at 280 nm and the UV-Vis CUPRAC derivatized antioxidant profile at 450 nm (displayed in the negative direction for visual inspection purposes only). Flowrate 4 mL/min and segmentation ratio set at 60%.

Figure 3. RF high throughput screening of antioxidant activity for tea samples (a) Green, (b) Camomile, (c) Earl Grey and (d) English Breakfast. CUPRAC selective detection at 450 nm, flowrate 4 mL/min and segmentation ratio set at 60%.

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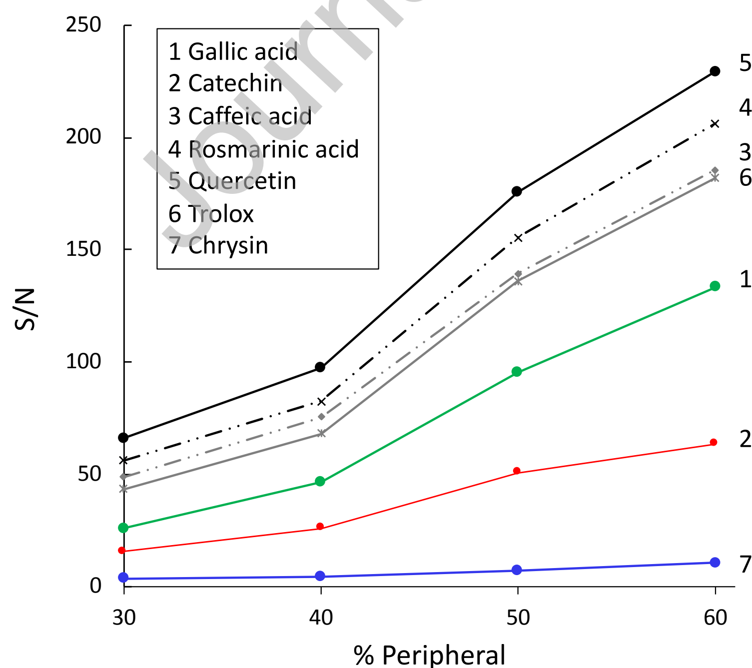
Figure 1

(a)



(b)

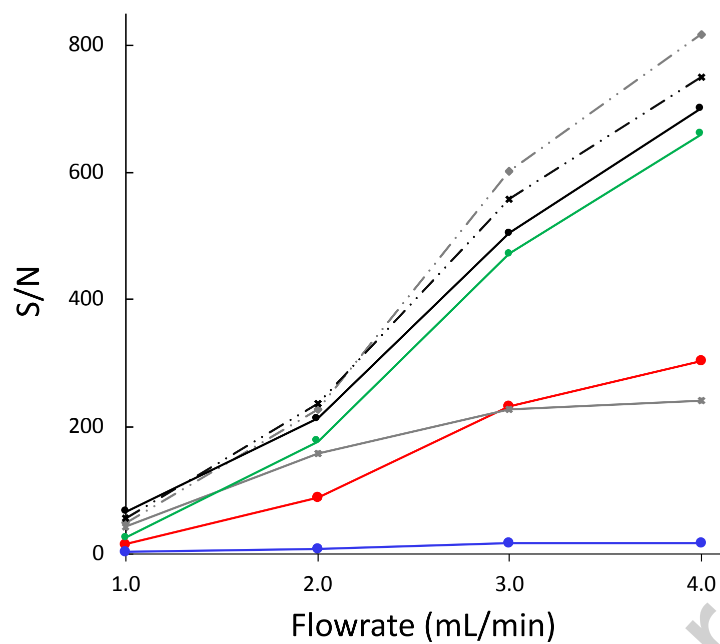


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Figure 2.

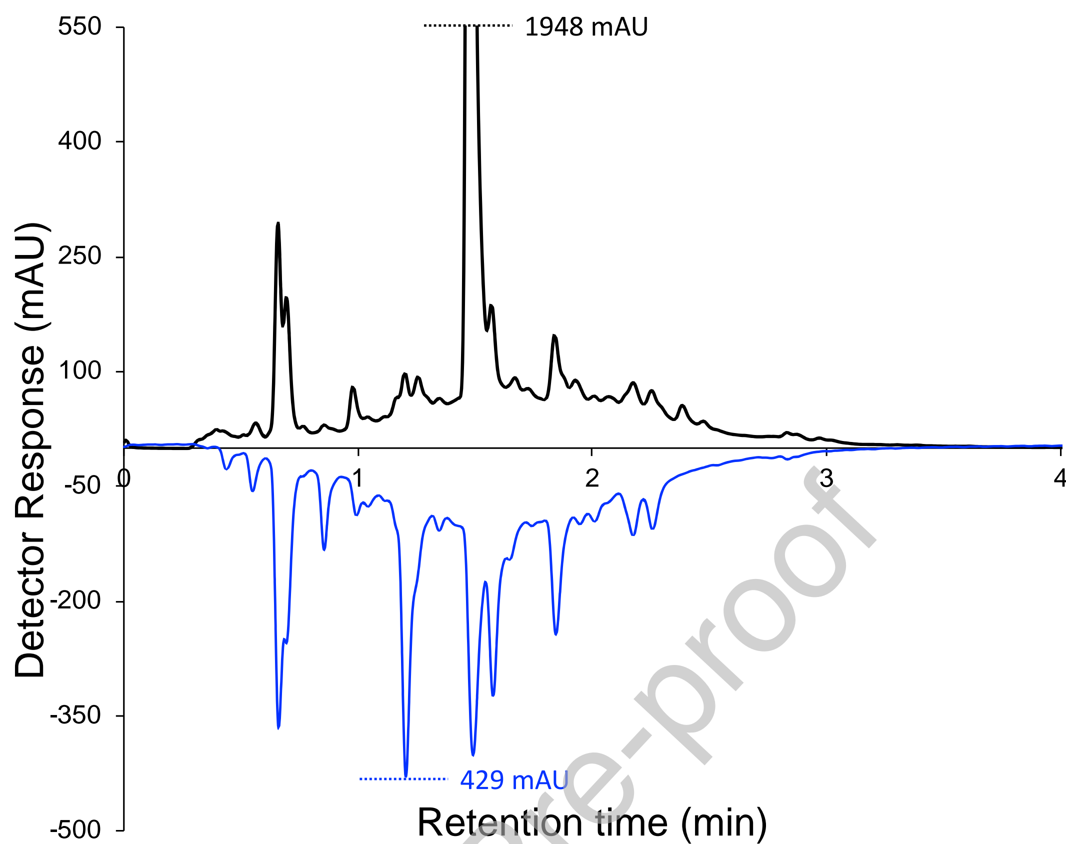

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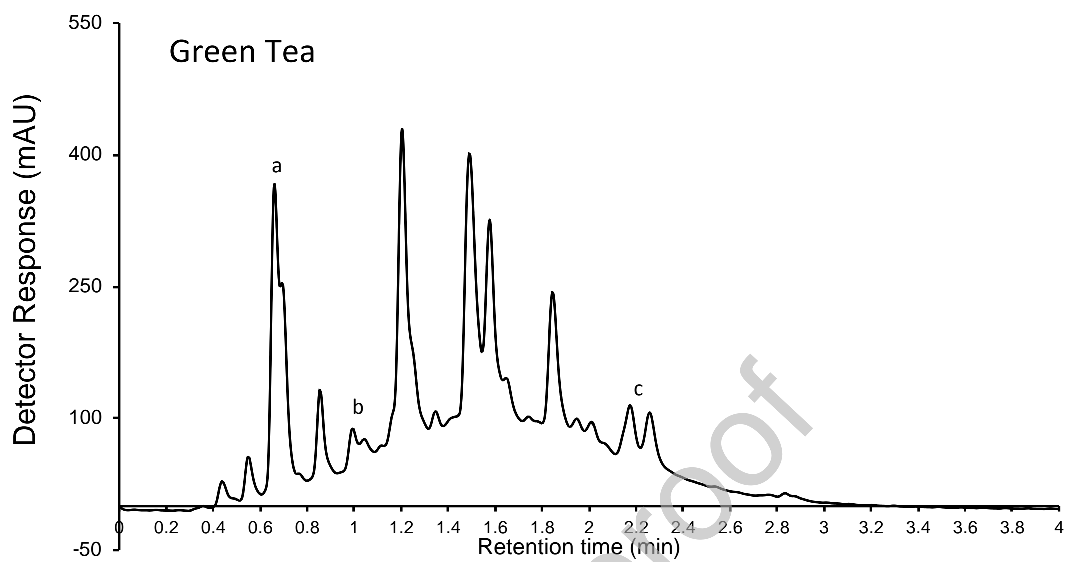
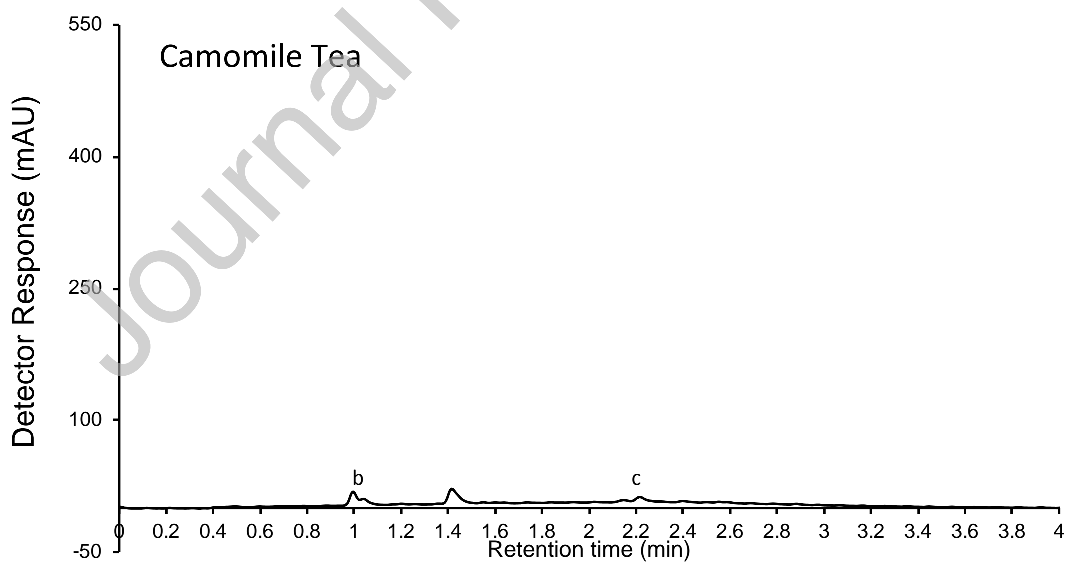
Figure 3.

(a)



(b)



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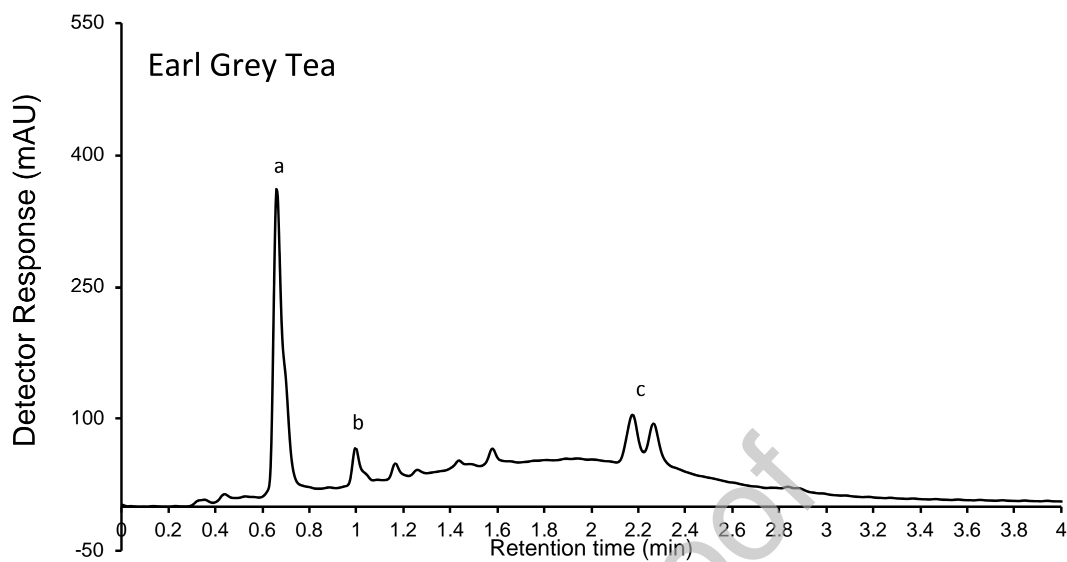
(c)



(d)

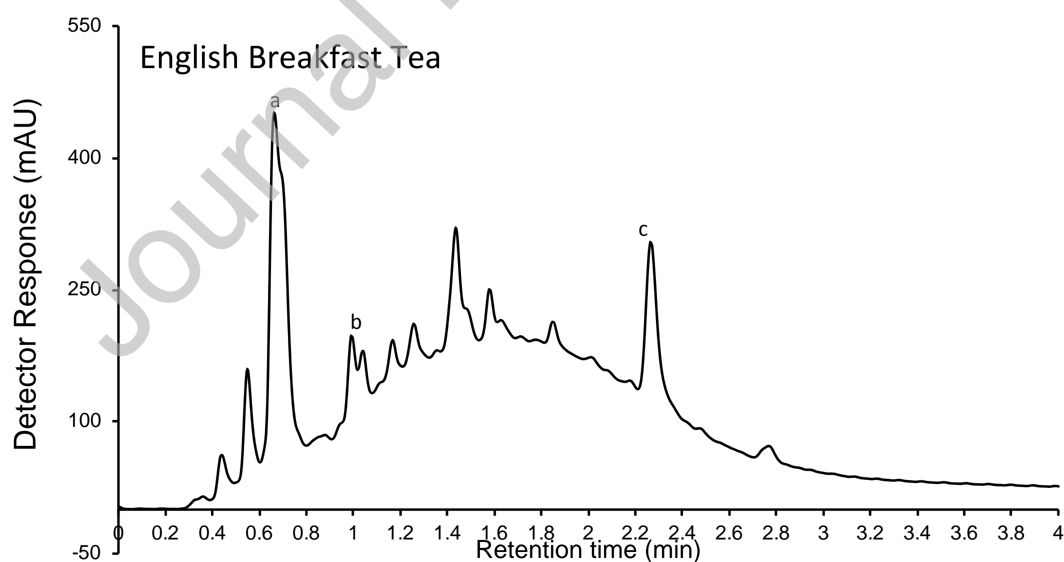


Table 1. Signal to noise (S/N), peak width, normalised peak width, retention time (RT) and normalised RT results with increased flowrate (FR) of the reaction flow column for the

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standards separation chromatograms performed on the RF column with a segmentation ratio of 60%, same peak numbering used in Figure 1.

S/N	**Peak number**						
FR (mL/min)	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1.0	26	16	49	56	66	43	4
2.0	177	88	228	237	213	158	8
3.0	471	232	601	559	504	228	17
4.0	660	304	818	750	701	241	17
Width							
FR (mL/min)							
1.0	0.91	0.96	0.95	0.85	0.97	0.76	0.37
2.0	0.49	0.61	0.47	0.37	0.38	0.37	0.25
3.0	0.35	0.39	0.32	0.27	0.30	0.27	0.13
4.0	0.26	0.28	0.27	0.25	0.25	0.20	0.14
Normalised width							
FR (mL/min)							
1.0	0.91	0.96	0.95	0.85	0.97	0.76	0.37
2.0	0.98	1.22	0.94	0.74	0.76	0.74	0.50
3.0	1.05	1.17	0.96	0.81	0.90	0.81	0.39
4.0	1.04	1.12	1.08	1.00	1.00	0.80	0.56
SD (min)	*0.06*	*0.11*	*0.07*	*0.11*	*0.11*	*0.03*	*0.09*
RT							
FR (mL/min)							
1.0	2.69	5.04	6.33	9.55	11.04	12.47	14.25
2.0	1.33	2.53	3.15	4.81	5.57	6.25	7.18
3.0	0.87	1.66	2.06	3.17	3.66	4.12	4.73
4.0	0.65	1.25	1.54	2.39	2.76	3.10	3.57
Normalised RT							
FR (mL/min)							
1.0	2.69	5.04	6.33	9.55	11.04	12.47	14.25
2.0	2.66	5.06	6.30	9.62	11.14	12.50	14.36
3.0	2.61	4.98	6.18	9.51	10.98	12.36	14.19
4.0	2.60	5.00	6.16	9.56	11.04	12.40	14.28
%RSD	*1.61*	*0.73*	*1.36*	*0.48*	*0.60*	*0.51*	*0.50*

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Conflict of Interest

All authors declare no conflict of interests.

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