


# Antioxidant Profiling of Ginger via Reaction Flow Chromatography

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## Abstract

Reaction flow (RF) chromatography is a powerful and efficient approach that utilizes conventional high-performance liquid chromatography (HPLC)–ultraviolet (UV)–visible detection. This technique exploits a novel column end-fitting and an extra HPLC pump that delivers a reagent specific for selective detection, in particular the antioxidant profiling of natural products. This study employed RF for the first time to identify antioxidants in a commercial ginger sample. This demonstrated the previously validated assay's ease and power to extract information about the natural product's antioxidant properties. Due to the simplicity involved with data analysis and peak matching process, the following information was revealed between the chemical and antioxidant profiles: three of the strongest antioxidant activity peaks in the ginger sample (593 nm) did not correlate with the three most abundant chemical profile peaks (UV absorbance at 254 and 280 nm); the ratio of seven antioxidant peaks may be potentially used for food authenticity purposes, and future research should target these peaks for the early discovery of novel antioxidants sourced in ginger. Utilization of this previously validated assay provided the resolution of numerous peaks in the ginger extract and information associated with their antioxidant attributes and chemical abundance. This approach is more informative than total antioxidant assays that lack compound specificity information. Furthermore, it is superior to mass spectrometric (MS) assays that cannot evaluate each compound's antioxidant strength, and does not involve the expense involved in the acquisition and maintenance of the MS detection hardware, and does not require the high level of expertise needed to conduct the MS data analysis.

## Keywords

antioxidant, reaction flow chromatography, ferric reducing antioxidant power assay, ginger, post column derivatization, selective detection

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## Introduction

Reaction flow (RF) chromatography exploits high-performance liquid chromatography hyphenated with online post column derivatization (PCD) selective detection.<sup>1</sup> It is more powerful and efficient than conventional PCD methods in terms of the observed separation efficiency and signal-to-noise ratio.<sup>1</sup> A previous study by our group validated an RF technique for the analysis and quantitation of several antioxidant molecules (Trolox, rosmarinic acid, chlorogenic acid, caffeic acid, rutin hydrate, and quercetin) using the ferric reducing antioxidant power (FRAP) assay that relies on the ability of antioxidants to reduce iron (III) to iron (II).<sup>2</sup> This previously validated RF FRAP approach and RF PCD strategy have since demonstrated their application for the analysis of tea and coffee samples in comparison to the detection selectivity of a previously validated RF phenolic assay, and a validated cupric reducing antioxidant capacity (CUPRAC) alternative RF antioxidant assay, and has

been developed into a high-throughput qualitative <2 min RF FRAP assay.<sup>1–5</sup>

The popularity and consumer interest in functional foods such as teas and coffees continue to increase due to their potential well-being functions, including the ability to arrest the effect

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of free radicals in the human system *via* antioxidants. The rhizomes of *Zingiber officinale* (ginger) are one of the most popular functional foods, and commonly used as a flavor and/or nutritional additive in the food and beverage industry. Several studies have been conducted to show the effective antioxidant properties of ginger (from different sources) via benchtop ultraviolet (UV) spectrophotometric antioxidant assays that utilize the FRAP and 2,2-diphenyl-1-picrylhydrazil (DPPH) reagents.<sup>6</sup> For example, the ethanol extract of ginger collected from Kathmandu, Nepal, resulted in an antioxidant FRAP value of 3.86 mM/100 g.<sup>7</sup> It has also been demonstrated that phenolic compounds were the main contributor of antioxidant activity in ginger and turmeric extracts.<sup>8</sup> Furthermore, the ferric FRAP activity of the rhizomes was higher than that of the leaves in 2 varieties of Malaysian young ginger.<sup>9</sup> Individual bioactive compounds from ginger have been subjected to antioxidant assays to investigate the key compounds that contribute to the overall antioxidant activities, and 6-gingerol was revealed to correlate strongly with the high FRAP-reducing activity.<sup>10</sup> However, over 400 different chemical compounds have been identified in the ginger extract and the 'offline' benchtop UV spectrophotometer FRAP assay is only designed to analyze the total antioxidant activity.

To solve this issue, the bulk sample's total antioxidant response is often further elucidated with more comprehensive analytical techniques such as 'fingerprinting'/chemical profiling and structure elucidation using liquid chromatography-ultraviolet (LC-UV), liquid chromatography with tandem mass spectrometry (LC-MS/MS), and nuclear magnetic resonance (NMR) spectrophotometry to search for and identify the key bioactive compounds.<sup>11-16</sup> Such approaches are expensive in terms of labor, consumables, instrumentation, and data analysis, and cannot provide information associated with each peak's antioxidant strength. To measure each specific peak's antioxidant power, fractionation of the sample must be undertaken, followed by testing for the antioxidant activity using benchtop UV spectrophotometric assays.<sup>17</sup> This is a very laborious process and there is a risk of losing activity prior to testing. In contrast, the 'online approach' utilizing PCD shows the antioxidant response of each separated compound/peak in the samples in an efficient manner. The previously validated RF FRAP approach provides useful guidance on the selection and identification of the most potent antioxidants in the sample, along with a profile that illustrates each sample's complex chemical fingerprint. The antioxidant profile obtained by RF FRAP can be used as a guide for targeted structure elucidation studies of the antioxidant peaks, a more simplistic approach than untargeted approaches that are not selective for antioxidants.

In this study, we demonstrated the ease and specificity of antioxidant profiling of ginger for the analysis of a commercially available ginger sample on the Australian market. This study serves to illustrate the correlation of separated peaks/compounds in ginger with their antioxidant response, which is potentially useful for early discovery of the key antioxidant peaks of ginger and/or food authenticity applications.

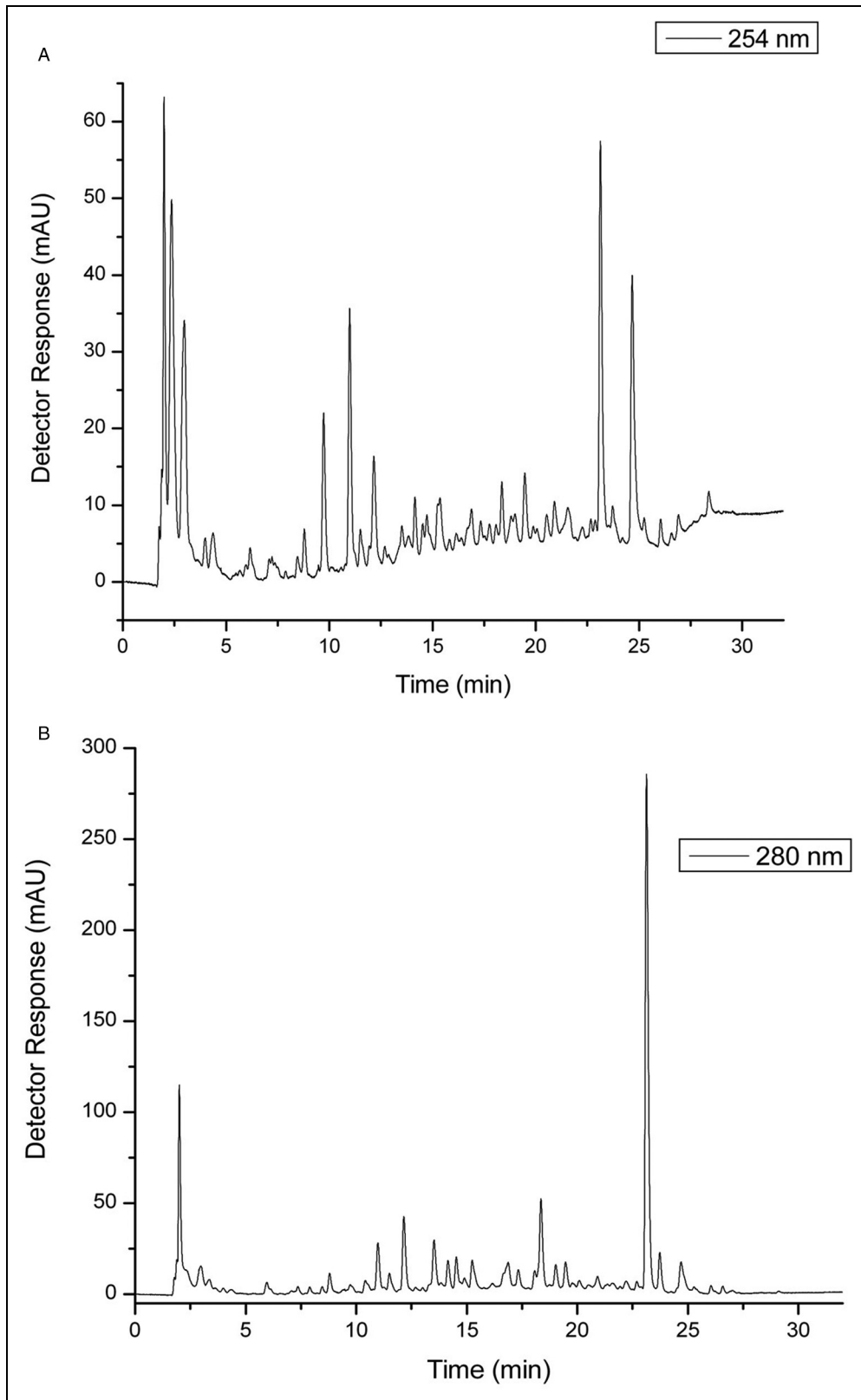
## Results and Discussion

The UV chromatograms of ginger obtained at 254 and 280 nm are illustrated in Figure 1(a) and (b), respectively. A large number of peaks were resolved in the separation space that illustrated both the complexity and abundance of the compounds in the ginger extract. The chemical fingerprint serves as a preliminary "chemical signature". Albeit, an expansion of the separation space, for example using multidimensional high-performance liquid chromatography (HPLC), or a reduction in the number of components displaced in the separation space via selective detection, provides a higher quality chemical signature. However, the chromatographic data obtained using just UV detection does not reveal any information on the correlation with antioxidant activity. Thus, incorporating an antioxidant assay that is performed simultaneously with an HPLC separation, via a PCD reaction enables the assignment of the UV peaks to that of the "active" antioxidant peaks. Conventional PCD processes, however, suffer from sensitivity and resolution issues associated with the relatively large inefficient mixing post-column volumes required for their approaches.<sup>18-20</sup>

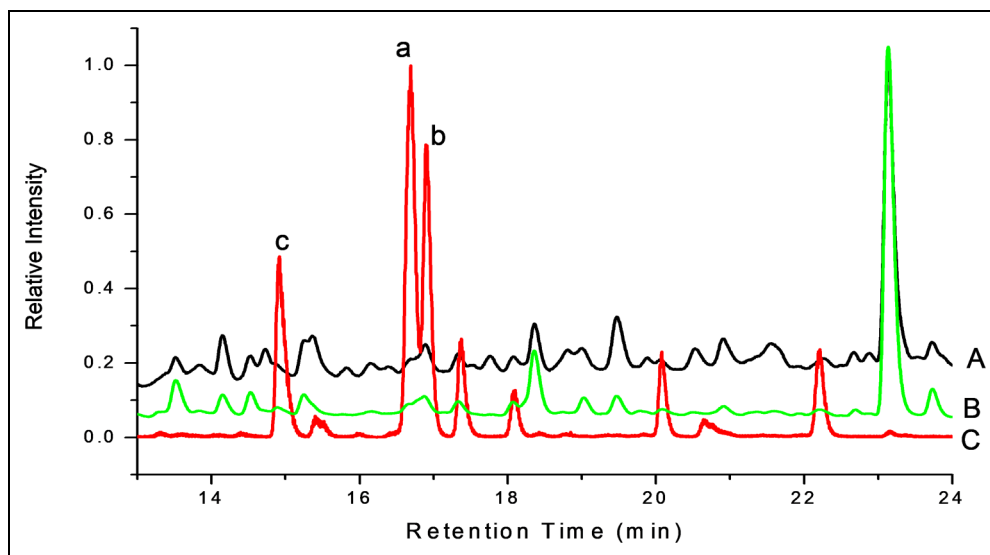
The chemical abundance profile, along with the antioxidant profile of the ginger extract using the previously validated RF FRAP process is shown in Figure 2 (traces A–C). All antioxidants were observed in the chromatogram between 14 and 23 min; hence, only this region of the separation has been illustrated. To correlate the chemical abundance with the associated antioxidant response, the chromatographic profiles of the ginger observed at 254 nm (trace A) and 280 nm (trace B) are included in Figure 2. For visual clarity, all chromatograms were normalized to the most intense signal response in each respective chromatogram, and traces A and B were offset by 10% and 5%, respectively.

Among all the separated peaks, seven peaks with strong FRAP antioxidant response were >10% of the maximum observed signal response; three additional antioxidant peaks were detected with responses <10%. Such information may be useful for food authenticity purposes. For example, a combination of the RF profile attributes to the identity, and confirms that no alteration/adulteration has occurred to the commercial functional food with respect to the antioxidant profile's number of peaks, their retention times, and the ratio of their responses.

The most interesting aspect of the RF antioxidant profiling was that the major components detected using UV did not result in a response to the FRAP reagent. Hence, all of the detected antioxidants were minor components in the UV chromatograms. The largest of these antioxidant peaks did not exceed even just a few percent of the most abundant peaks in the UV chromatograms. This is an extremely important piece of information provided by the previously validated RF FRAP approach to be revealed about ginger, as the search for the components responsible for the antioxidant activity would have likely been misguided and focused on the major components, rather than the relatively minor ones. Hence, the RF



**Figure 1.** Chromatographic profile of ginger with ultraviolet (UV) detection at (a) 254 nm and (b) 280 nm.



**Figure 2.** Chromatographic profiles for the antioxidants that responded to the FRAP reagent for ginger with a colorimetric response at 593 nm (trace C), compared to the UV absorption at 254 nm (trace A) and 280 nm (trace B). All chromatograms were normalized to the height of the band that displayed the highest response in each respective mode of detection. Compounds a, b and c are highlighted as they had weak UV responses and large antioxidant responses.

Abbreviations: FRAP, ferric reducing antioxidant power; UV, ultraviolet.

FRAP approach applied to ginger for the first time empirically highlights that the most active antioxidant chemicals in ginger are the minor, unknown compounds. It is worth mentioning that the UV response does not directly relate to the concentration of any particular component since there is no information on the absorptivity coefficients of each component.

Compounds 'a' and 'b' in Figure 2 are of particular interest. These were the strongest responding antioxidants with the highest peak heights detected using PCD. However, the UV response of these 2 peaks was not prominent in traces A and B. This highlights that compounds 'a' and 'b' may not be of high value in ginger or have high UV absorbance within the detected wavelength. Compound 'c' showed a strong antioxidant response, but almost no response was detected with UV at 254 nm, but a minor peak was apparent at 280 nm. Importantly, compounds 'a' and 'b' were barely separated, and yet following detection using PCD the resolution of these 2 compounds was not lost. In all likelihood, had a conventional approach to PCD been employed, the extra column dead volume associated with the reaction loop would have resulted in the peak band broadening, resulting in loss of separation with both peaks coeluting.<sup>20</sup> Further studies are warranted to investigate the identity of compounds a, b, and c to confirm our findings.

A DPPH<sup>•</sup> antioxidant activity value for the ethanol extract of the ginger sample separated and profiled in this study was also obtained. The ginger extract had a total antioxidant capacity of  $60.9 \pm 2.5$  mg GAE/g DE. This result, achieved by the bench top 'offline' UV spectrophotometer assay, represents the bulk/total antioxidant response, but lacks specific information related to which specific antioxidants in the extract contribute to this result. Without further testing on fractions and

compounds, the mechanisms of the antioxidant activity are unknown. Furthermore, the quality of the plant extract collected from different sources varies largely,<sup>18</sup> which remains a major challenge concerning quality control and evidence-based efficacy study of medicinal plants.

The RF antioxidant profile can be undertaken following just a single injection of the sample since derivatized and underivatized flow streams can be monitored simultaneously.<sup>4</sup> However, it was not undertaken in our study as a second UV detector was not available. In this case, a repeat injection was employed with the PCD reagent turned off to provide an absolute confirmatory response of the antioxidants relative to the native underivatized state. The profiling of ginger by this previously validated RF FRAP approach provided critical information that can guide future targeted MS experiments to confirm the identity and quantity of the minor unknown antioxidant peaks. In particular, compounds a, b and c in Figure 2 could be the target for a structure elucidation study associated with the early discovery of novel bioactive compounds in ginger.

In summary, future natural product applications that can exploit the separation and subsequent activity testing *via* RF FRAP include establishing a ratio profile of the antioxidants, which could be used to monitor changes in the sample over time or through processing for QC/QA verification checks of the sample stability, and/or to detect for any adulteration or significant changes in the raw material. Such assays often use expensive selective detection techniques eg mass spectrometry, which does not obtain any antioxidant activity information of the separated peaks. Hence, in this short communication we demonstrate the ease of antioxidant profiling of ginger *via* the RF antioxidant profiling approach; data analysis via a simple

peak matching process; and highlight potential studies/applications of the significantly useful information obtained, in particular the use of the RF FRAP profiling results for guided structure elucidation experiments. These results should encourage the future implementation of this RF FRAP profiling approach for other natural products.

## Conclusion

This study demonstrated the power and simplicity of antioxidant profiling of ginger *via* a previously validated instrumental analytical method that exploited RF chromatography. Among all the separated peaks, seven displayed strong antioxidant responses >10% of the maximum observed signal response. Three of these had strong antioxidant responses to FRAP, but they did not represent the most abundant species with low UV absorbance at the detection wavelengths of 254 and 280 nm. This study highlighted the power of this antioxidant selective assay; an alternative to benchtop UV spectrophotometric “offline” total antioxidant assays that can only represent the bulk sample. Furthermore, it represents an alternative to selective detection compared to mass spectrometric assays that cannot discern the strength of antioxidant activity for each peak, are expensive to conduct in terms of acquiring and maintaining the MS detection hardware, and require higher expertise for data analysis. The findings of this study may support future early discovery research of ginger’s antioxidant compounds and/or food authenticity of ginger-manufactured products. Furthermore, it may encourage future implementation of RF as a simple yet information-rich approach to gain first insights and/or valuable information on natural products.

## Material and Methods

### Chemicals

Mobile phase solvents were all HPLC grade. Methanol and ethanol (AR grade) were purchased from Thermo Fisher Scientific (Australia) and Chem Supply Pty Ltd (Australia), respectively. The Ultrapure Milli-Q water (18.2 M $\Omega$ ·cm) was prepared in-house and filtered through a 0.22  $\mu$ m filter. Gallic acid, 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 (Australia). MediHerb™ Ginger liquid extract (prepared from dried *Z. officinale* Roscoe rhizome, dried extract: raw material 1:2, 90% aqueous ethanol) was obtained from Integra Healthcare Pty Ltd (Australia).

### Reagents and Sample Preparation

The FRAP reagents were prepared according to the protocol outlined by Benzie and Strain.<sup>18</sup> 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 adjusted to 3.6 ( $\pm$ 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 derivatization reagent, FRAP, was prepared daily and filtered through a 0.22  $\mu$ m filter prior to use.

### Plant Material Preparation

MediHerb™ Ginger liquid extract was evaporated and freeze-dried for 24 h. The dried extract was then redissolved in methanol to a concentration of 50 mg/mL and stored at a temperature of  $-80$  °C prior to use. Before HPLC analysis, an aliquot of the methanol solution was diluted 1:4 with water and passed through a 0.22  $\mu$ m nylon syringe filter.

### RF Chromatography Selective Detection of Antioxidants and Chemical Profiling Instrumentation

All analyses were performed on a Shimadzu HPLC System, equipped with a Shimadzu SCL-10Avp controller, a Shimadzu SIL-10AD vp auto injector, a Shimadzu LC-20AD pump, a Shimadzu FCV-10AL vp switching valve, a Phenomenex degasser DG-4400 degasser and a Shimadzu SPD-M10A vp detector. An additional Shimadzu LC10ADvp pump, fitted with an inline degassing unit (Phenomenex DG-4400) was used to deliver the PCD reagent.

### Chromatographic Conditions

The chromatographic conditions are based on the FRAP RF instrumental analysis method previously validated.<sup>2</sup> The HPLC separations were carried out using a Hypersil GOLD column (150  $\times$  4.6 mm, particle diameter 5  $\mu$ m) for RF analysis, supplied by ThermoFisher Scientific. The ginger sample was analyzed under reversed phase gradient conditions. The sample and column temperature were kept at 4 °C and 20 °C, respectively. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Gradient conditions optimized for peak capacity were as follows: 0 to 30, 5% B; 30 to 34 min, 100% B, and returned to 5% B for the last 0.1 min. The column flow rate was set at 1.0 mL/min.

The RF FRAP instrumental analysis method has been fully validated and published in our previous study.<sup>2</sup> Briefly, the mobile phase (1.0 mL/min) carried the injected sample solution (injection volume at 10  $\mu$ L) to the RF column for the separation. Then the FRAP reagent was delivered to the multipoint outlet of the RF column at a constant flow rate of 0.5 mL/min that

directed to 3 peripheral ports with the segmentation ratio between the central and peripheral ports set at 50:50. The central port was directed to either the fraction detector or waste. A second peripheral port directed the derivatized eluent to the PDA detector at wavelengths of 254 and 280 nm with the signal response obtained at 593 nm. Thus, 2 modes of detection were employed for both chemical and antioxidant detection using underivatized UV and PCD (visible). The third peripheral outlet was blocked. In addition, the samples were also analyzed with the PCD reagent pump turned off.

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### Declaration of Conflicting Interests

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