High Efficiency Post Column Derivatisations of Natural Products using Reaction Flow High Performance Liquid Chromatography

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Native Australian Food Plants are well known for their high antioxidant activity. High performance liquid chromatography (HPLC) coupled with post-column derivatisation (PCD) is a useful tool for the fast and efficient screening of antioxidants in complex mixtures. However, PCD usually involves the use of large reaction coils that jeopardise the separation performance gained on the HPLC column. Thus, a new and alternative technique for HPLC-PCD assays was employed, known as Reaction Flow (RF) chromatography, for the analysis of antioxidants and phenolic compounds in extracts from Backhousia citriodora leaves using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) for the analysis of antioxidants and a dual component reagent for the analysis of phenols comprising of potassium ferricyanide and 4-aminoantipyrene. RF chromatography not only provides a platform for efficient mixing to take place but does so without compromising separation performance. In this study, a number of antioxidant and phenolic compounds were detected using RF-PCD.

1. Introduction

There are countless known Native Australian Food Plants (NAFPs) that have served the indigenous community of Australia as food and medicine for thousands of years [1-3]. As interest in the health benefits of NAFPs has grown in the past decade, the nutritional composition of many plants have been investigated [4]. However, there are still many NAFPs commonly used by indigenous people where very little is known about the nutritional composition [5,6]. A range of NAFPs have been found to contain greater antioxidant content than common fruits, such as, blueberries. The antioxidant and phenolic content of twelve NAFP fruits was investigated by Netzel et al. [1,2], five of which exhibited 3 to 5 fold greater Trolox equivalent antioxidant capacity (TEAC) than that of blueberries, and six of which totalled Folin-Ciocalteu phenolic levels 2.5 to 4 times greater than blueberries [2,7]. Thus, it is evident that NAFPs are a rich source of antioxidant and phenolic compounds [7,8].

Most antioxidant and phenolic assays involve complex sample preparation, derivatisation reagents, such as, Oxygen Radical Antioxidant Content (ORAC), Ferric Reducing Ability of Plasma (FRAP), DPPH• (2,2-diphenyl-1-picrylhydrazyl) and ABTS• (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and analysis by UV absorbance. Such methods when applied at the bench on the bulk sample can only give information about the total antioxidant content and no information gained is about the chemical composition of the samples. For the determination of the presence of antioxidants and their identification other analytical techniques, such as, HPLC with post column derivatisation and HPLC-MS must be employed. These methods can be labour intensive and time consuming, thus, efficient and rapid methods for the screening of antioxidant and phenolic content in complex mixtures is always sort [7].

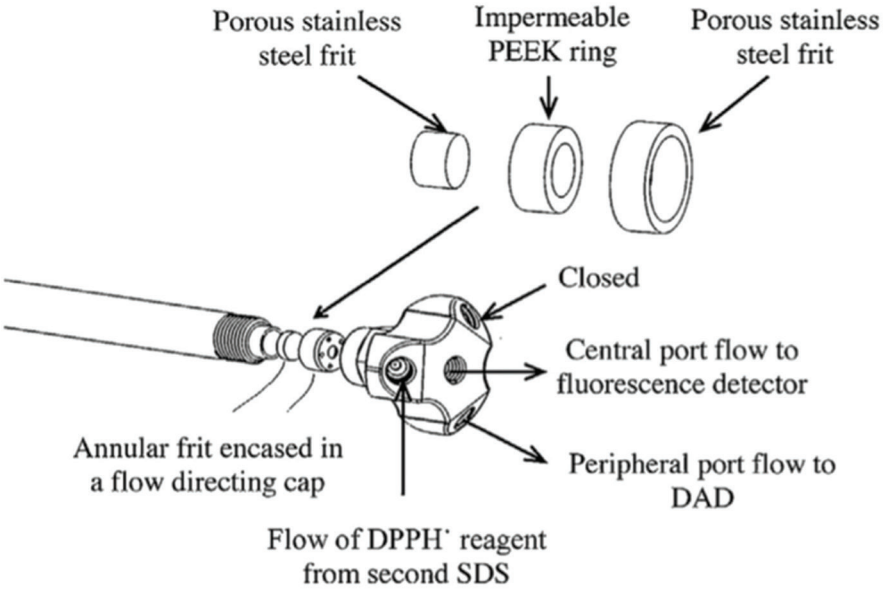
Post-column derivatisation (PCD) methods of detection are useful for the natural product chemist because they enhance the information that is gained from separations performed in HPLC. Not only do they provide detection, but the derivatisation process can be specific to functional aspects of a molecule, which then can be related to its bioactivity. Despite these benefits, PCD is hindered by large post-column dead volumes arising because of the need to the use reaction coils, subsequently causing band broadening and loss of separation power [9]. A new technique for efficient PCD analyses has been developed using Active Flow Technology (AFT) columns in Reaction flow (RF) mode [9-12].

Reaction flow chromatography columns employ a special purpose-built four-port end-fitting and a three piece annular frit containing a central porous region, separated from an outer porous region by an impermeable ring. The radial central flow region of the eluent exits the column via a radial central exit port, while the flow near the wall region exits the column via any of the three ports that align with the outer peripheral porous region of the frit.

Therefore the wall flow region and the central flow region are isolated from each other [10]. The portion of flow in the wall region, relative to the central flow region

can also be varied, and depends upon the relative pressure differential between the radial central exit port and the peripheral ports, and the characteristics of the outlet frit. In this column design it is important to emphasise that the wall flow and radial central flows are separated by the impermeable barrier in the annular frit design. An understanding of this basic function is important since post column derivatisation reagents can be added into the flow stream through one or two of the outlet ports that align with the outer porous region of the frit, rather than using Tee pieces in post column configurations [10-12]. These reagents then mix with the mobile phase and sample that leaves the HPLC column inside the frit. The mixing process is very efficient, often negating the use of reaction coils that are normally required to mix reagents with the sample. Furthermore, since the radial flow stream is separated from the wall flow region, the central flow is not subjected to the post column derivatisation reagents and thus 'native' sample leaves the column through this single port and can be detected using a detection process separate from the derivatisation method. Therefore, multiplexed detection enables a greater understanding of the sample characteristics [7]. Since no reaction coil is required, or at least the volume of the reaction coil can be greatly reduced, separation efficiency is not compromised through the band broadening that takes place in large reaction coils [13]. The diagram above illustrates the application using the antioxidant detection process employing DPPH reagent. Peaks are therefore sharper and depending on the type of chemical derivatisation and detection process, the signal intensity is often increased, even despite the shorter period of time required to undergo the derivatisation process and the fact that only a small portion of the sample is subjected to the post column derivatisation. More often than not, the background noise is decreased, so that even when, or if, the signal intensity is decreased, the actual specific sensitivity (factoring in S/N) is increased [13-17].

The combination of RF and multiplexed detection has the potential to yield a significant amount of information about the nature of the sample of interest in half the time it would otherwise take if the analyses were undertaken separately in the conventional manner [7]. RF chromatography has also been found to minimise the complexity in PCD preparation time as well



as instrumental setup [11,12,16], such as in the case of PCD reagent, fluorescamine, for the analysis of amino acids [11]. The use of a RF column reduced the number of pumps, coils and mixing devices that are otherwise required in conventional HPLC-PCD analyses using fluorescamine [11]. Ultimately, the RF column enables the complete removal of post column reaction coils, providing high efficiency separation with specific detection.

In this study, RF was utilised to analyse the bioactivity of NAFPs (specifically, lemon myrtle (LM) (Backhousia citriodora)) using ABTS• and phenol reagents – 4-aminoantipyrene and potassium ferricyanide, to provide insight to its antioxidant and phenolic content of leaf extracts.

2. Experimental

2.1 Chemicals and Reagents

HPLC grade mobile phase solvents (Themo Fisher Scientific, North Ryde NSW Australia.) were used. Milli-Q water was prepared in house using a 0.2 µm filter (Ultrapure, Millipore, Kilsyth, VIC Australia.). Ammonium acetate, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and potassium persulfate were purchased from Sigma Aldrich (Castle Hill, NSW Australia.). 4-aminoantipyrine was purchased from ACROS Organics (Geel, Antwerp, Bel.) and potassium ferricyanide was purchased from AnalaR (Poole, Dorset, UK).

Solutions containing 0.3 mg/mL of ABTS and 10 mg/mL sodium persulfate were prepared in Milli Q water and sonicated for 10 minutes to dissolve. The 4-aminoanitpyrene and potassium ferricyanide reagents were both prepared at a concentration of 1.5 mg/mL in 0.1 M ammonium acetate (pH 9) and sonicated for 10 minutes to dissolve.

Mature lemon myrtle (LM) leaves were harvested from established trees grown in an uncontrolled environment at Muru Mittigar culture centre gardens in Castlereagh, NSW Australia. The freshly harvested leaves were pounded separately in either Milli-Q water or methanol using a ceramic mortar and pestle. A total of 20 mL of solvent was added per gram of sample. The samples were sonicated for 5 min at room temperature and filtered using a 0.22 µm nylon filter [7].

2.2 Instrumentation and Chromatographic Conditions

2.2.1 Column

Separations for RF were conducted using a HyPURITY C18 50 × 4.6 mm, 3 µm column with a 4-port outlet head-fitting (Thermo Scientific,Runcorn, Cheshire, UK). Conventional HPLC separations were conducted using a column of the same phase and dimensions with a standard column end-fitting.

2.2.2 Conventional UV-Vis Detection

The leaf extracts were analysed via UV-Vis detection using the conventional column. The chromatographic separations were carried out on a Shimadzu HPLC equipped with a Shimadzu Prominence LC-20AD

pump, a Phenomenex Degassex model DG-440 inline degasser unit and Shimadzu SPD-M10Avp PDA detector (254 nm). The chromatographic analyses were carried out using gradient elution with an initial mobile phase composition of 100% water with 0.1% formic acid running to a final mobile phase composition of 100% methanol with 0.1% formic acid, at a rate of 2% min^{-1}. The flow rate was set to 2 mL min^{-1} and injection volumes were 20 µL.

2.2.3 RF - ABTS Detection

ABTS detection was carried out in RF mode on the same instrument in section 2.2.2 Conventional UV-Vis Detection, with the same chromatographic conditions. The flow ratio between the peripheral port and central port was set at 50:50. Figure 1a illustrates the ABTS detection instrumental set up.

The ABTS and potassium persulfate solutions were both pumped at a flow rate of 0.5 mL min^{-1}. The two solutions were mixed at a zero dead volume t-piece before the resultant mixture was passed through a 1000 µL mixing loop (note: this mixing loop was employed to mix reagents only, prior to the introduction to the sample and column, i.e., at no time did the sample that was eluting from the column pass through this loop). The solution was then pumped into 1 peripheral port of the RF column. A second Peripheral port was connected to a PDA detector with the analysis wavelength set to 734 nm to monitor the excess ABTS$^{\bullet}$ within the eluent. The third peripheral port was blocked while the flow from the central port was allowed to flow to a collection vessel.

2.2.4 RF - Phenol Detection

The chromatographic experiments for phenol detection were undertaken on a Waters 600E Multi Solvent Delivery LC System, equipped with a Waters 717 plus auto injector, two Waters 600E pumps, two Waters 2487 series UV-Vis detectors and two Waters 600E pump controllers. An additional Shimadzu LC-10ATvp pump was used for the delivery of PCD reagents. Chromatographic separation was carried out using gradient elution with an initial mobile phase composition of 100% 0.1 M ammonium acetate buffer (pH 9) running to a final mobile phase composition of 100% methanol, at a rate of 2% min^{-1}. The flow rate was set to 2 mL min^{-1} and the injection volume was 20 µL. The flow ratio between the peripheral port and central port was set to 50:50. Figure 1b illustrates the phenol detection instrumental set up.

Phenol detection was achieved by introducing the 4-aminoantipyrene and potassium ferricyanide reagents into two of the peripheral ports of the RF column. The flow rate of the 4-aminoantipyrene solution was set to 0.5 mL min^{-1} and the flow rate of the potassium ferricyanide solution was set to 0.4 mL min^{-1}. The third peripheral port of the column was connected to a UV-Vis detector set to 500 nm to monitor the derivatised eluent. The central port of the column was connected to a second UV-Vis detector set to 254 nm to monitor the native (underivatised) effluent stream.

2.3 Data processing

Data analysis was undertaken using Origin and Microsoft Excel. For the reagent based detection methods (i.e. ABTS$^{\bullet}$ and phenol detection) the blank chromatographic profile was subtracted from the sample chromatographic profile.

3. Results and Discussion

The lemon myrtle leaf extracts were analysed for their bioactivity with respect to antioxidant and phenolic content. Water and methanol extracts of the leaves were analysed in RF using ABTS$^{\bullet}$ and phenol specific detection using 4-aminoantipyrene and potassium ferricyanide as PCD reagents. The phenol specific analysis was also multiplexed with a UV-Vis detector connected to the central port of the column to monitor the underivatised eluent, however, the ABTS$^{\bullet}$ analysis was not multiplexed. Since, the phenol detection method utilised a slightly different mobile phase compared to the ABTS$^{\bullet}$ analyses, a conventional UV-Vis analysis on the leaf extracts was carried out to account for the chromatographic difference between the two RF-PCD techniques. Figure 2 illustrates the chromatographic profile for the leaf extracts with conventional UV-Vis detection at 254 nm. A significant number of peaks were observed for each extract, with the majority of peaks eluting before 30 minutes. However, some peaks were still observed up to 50 minutes. The chromatograms of the lemon myrtle water and methanol extracts (Figure 2a and 2b, respectively) showed that the highest intensity peaks occurred after 5 minutes, with the intensity of the peaks being greater in the methanol extract, especially for the latter eluting components.

3.1 ABTS$^{\bullet}$ Detection

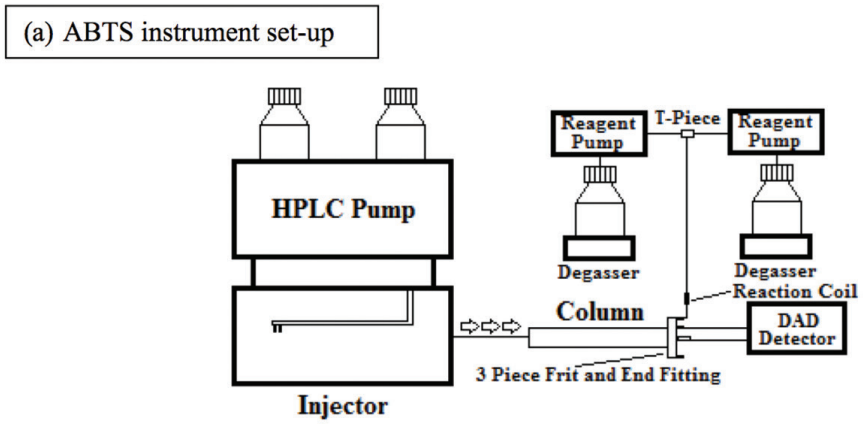


Figure 1a

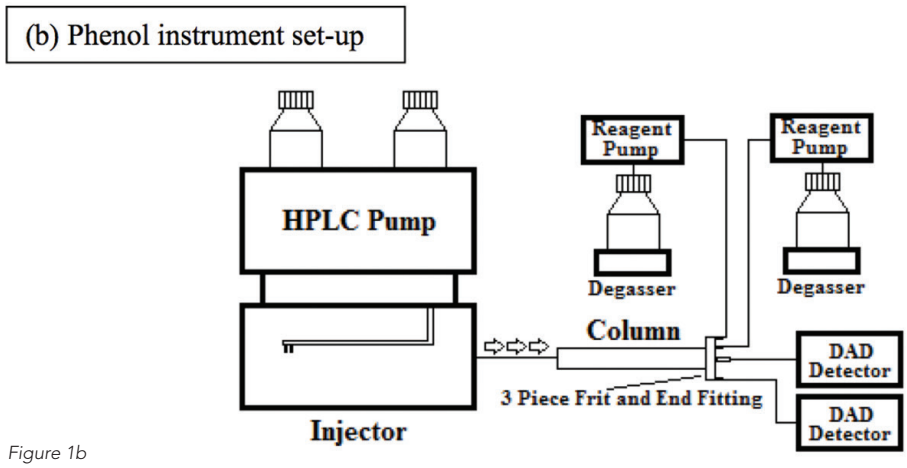


Figure 1b

ABTS• is a sterically hindered stable radical reagent [18] that is commonly used for colorimetric assays of antioxidants [19]. Analysis of the lemon myrtle extracts using ABTS• was performed in reaction flow mode, meaning that the reagent was pumped directly into the end fitting of the column where mixing and reaction could take place within the end frit of the column. The increased mixing efficiency resulted in the ability to remove the reaction loop between the mixing of the reagents and the detector and therefore the negative effects of dead volume. Additionally, conventional methods of ABTS• detection require lengthy reagent preparation time, which involves a waiting period of up to 20 hours of reaction time before use [20]. In the process of RF-PCD, detection was achieved with the waiting period effectively eliminated by mixing the reagents in real time just prior to its introduction to column effluent. Although, the RF-PCD technique may require further investigation to optimise sensitivity, nevertheless a significant number of ABTS• peaks were observed.

Figure 3 shows the chromatographic profile of the ABTS• detection response for each of the leaf extracts (3a being the water extract, 3b being the methanol extract). Interestingly, all peaks that gave a response to ABTS• eluted within the first 21 minutes, and notably, the two major components in the UV chromatograms that eluted at around 25 minutes gave no ABTS• response. The lemon myrtle water extract (Figure 3a) showed a cluster of peaks between 7 and 12 minutes (at least seven responding to ABTS•), which can also be seen in the UV-Vis chromatogram. The large peak at 8 minutes has four observable shoulders indicating the co-elution of multiple antioxidant compounds. The methanol extract (Figure 3b), shows a single large peak with a number of much smaller peaks appearing after 8 minutes.

The ABTS• response to the leaf extracts indicates that there are a number of antioxidant compounds present in these leaves. Water extraction gave a greater number of observable peaks compared to methanol extraction. However, a number of peaks in the methanol extract may have co-eluted in the single large peak close to the void. Besides the single large peak in the methanol extract, the rest of the peaks gave a signal intensity that was similar to that in the water extract. Differences in the chromatographic profile of the water and methanol extracts indicate that each of the solvents extract a different range of antioxidant compounds indicating that

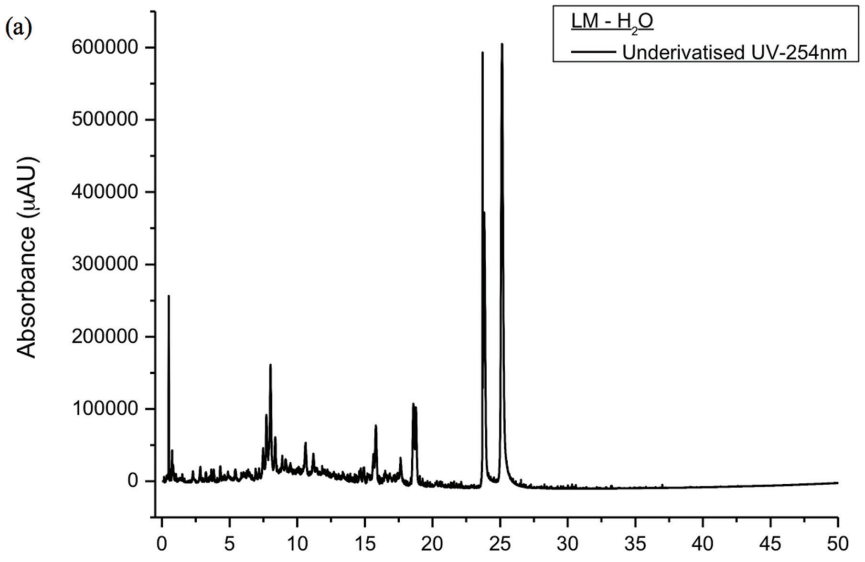


Figure 2a

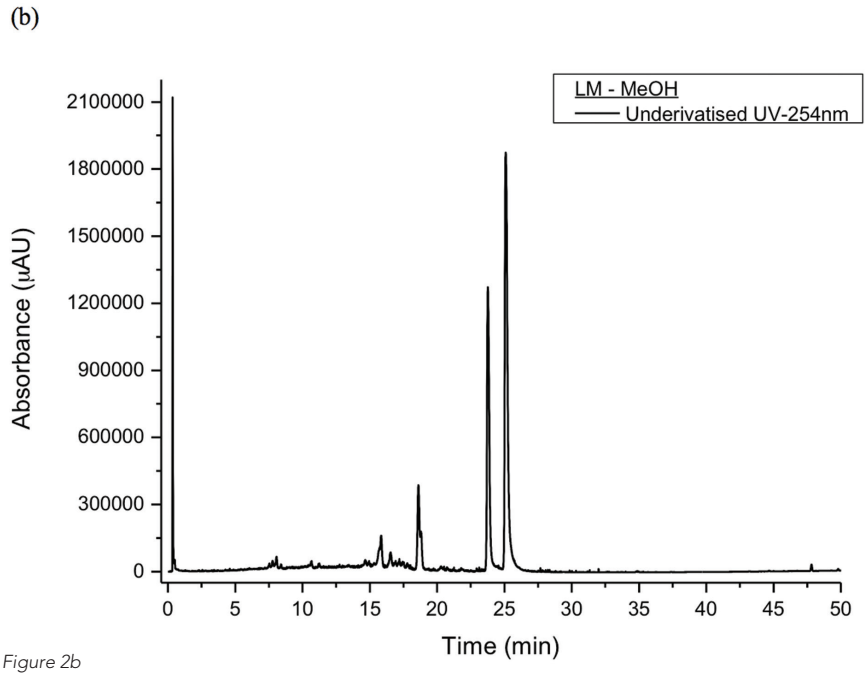


Figure 2b

no single solvent extraction procedure can provide a complete picture of the antioxidant profile of the sample. An obvious example of the difference in extraction efficiencies of each solvent could be seen in the colour of each solution. The methanol extracts showed an obvious intense green colour that was not present in the water extracts indicating that only the methanol was able to extract the chlorophylls from the leaves. The presence of the chlorophylls in the methanol extract may be the cause of the intense response around the void time in the methanol extract chromatograms as chlorophylls are organometallic molecules that may not be retained on the C18 stationary phase.

3.2 Phenol Detection

Detection using the phenol reagent was also carried out in RF mode. The central, underivatised flow was also monitored using a UV-Vis detector set to 254 nm. Figures 4 and 5 present the chromatograms, both derivatised and underivatised, collected using the phenol reagent method. All chromatographic profiles showed high correlation between the underivatised UV-Vis and phenol derivatised profiles suggesting that the extracts contain a number of phenol based compounds.

The lemon myrtle chromatograms (Figure 4 and 5) show a large underivatised UV-Vis response at the void time; however, the response of the phenol reagent was limited

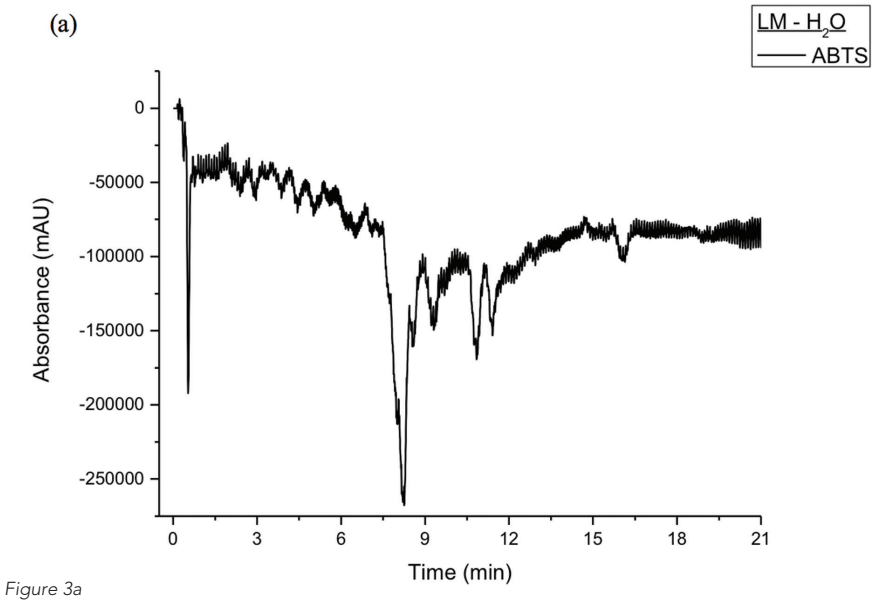


Figure 3a

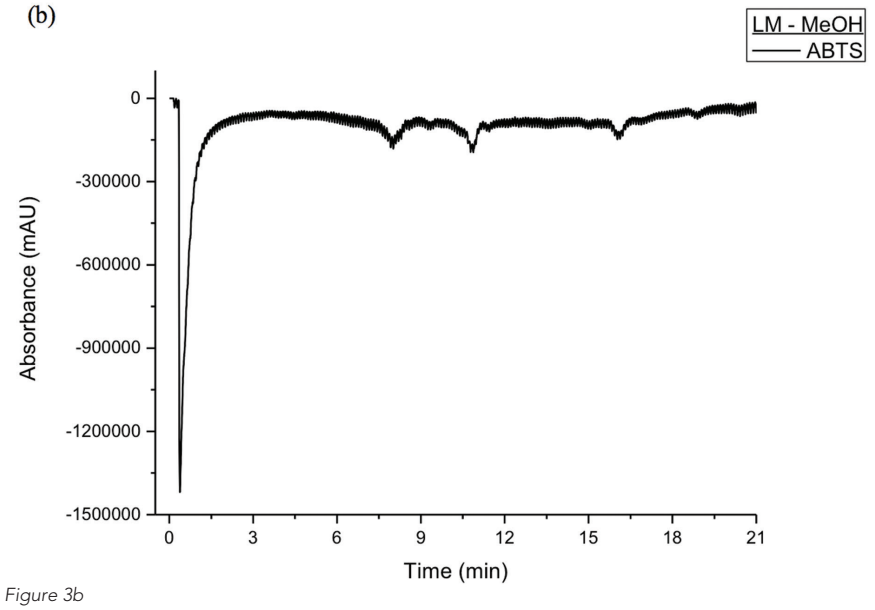


Figure 3b

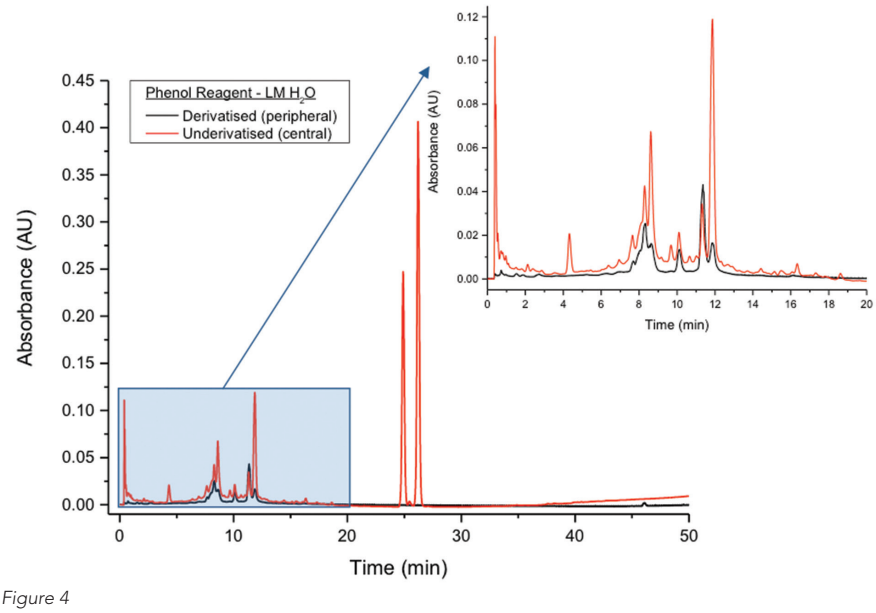


Figure 4

in the first 5 minutes of the chromatogram. This mirrors a trend that is also observed in the ABTS• antioxidant derivatisation method. A number of peaks with retention times of between 6 and 17 minutes show responses to both the underivatised UV-Vis and the phenol reagent detectors. The chromatographic profiles show variation in relative response factors between the underivatised UV-Vis and the phenol reagent chromatograms. Additionally, the phenol reagent and underivatised UV-Vis chromatograms show higher response for the methanol extract compared to the water extract and no compounds responded to the phenol reagent with retention times of more than 20 minutes. The two large peaks that were detected in the conventional UV-Vis analyses at a retention time of around 25 minutes were also detected in the underivatised stream in the phenol detection experiment with retention times of 24 and 26 minutes respectively. These compounds did not respond to either the phenol reagent or the ABTS• reagent, indicating a lack of both phenol functionality and antioxidant activity in these compounds.

Detection using the phenol reagent showed a number of similarities and differences compared to the ABTS• antioxidant detection method. Given that the mobile phase composition was different in both experiments and differences in the UV-Vis chromatographic profile have been noted, additional information needs to be collected to definitively state whether the antioxidants and phenolic compounds that were detected are the same or different compounds.

4. Conclusions

Bioactivity screening in NAFPs, such as the lemon myrtle leaves, is a time consuming and laborious process when carried out under conventional HPLC methods. The use of AFT-PSF columns enabled RF chromatography with PCD reagents and multiplexed detection of the underivatised central flow, allowing for fast and efficient antioxidant and phenolic screening. RF-PCD chromatography allows for the elimination of reaction coils typically utilised in most traditional PCD techniques and therefore more efficient separations, which is critical in the analysis of complex samples. Additionally, the multiplexed detection of derivatised and underivatised streams resulted in the collection of additional information from every injection.

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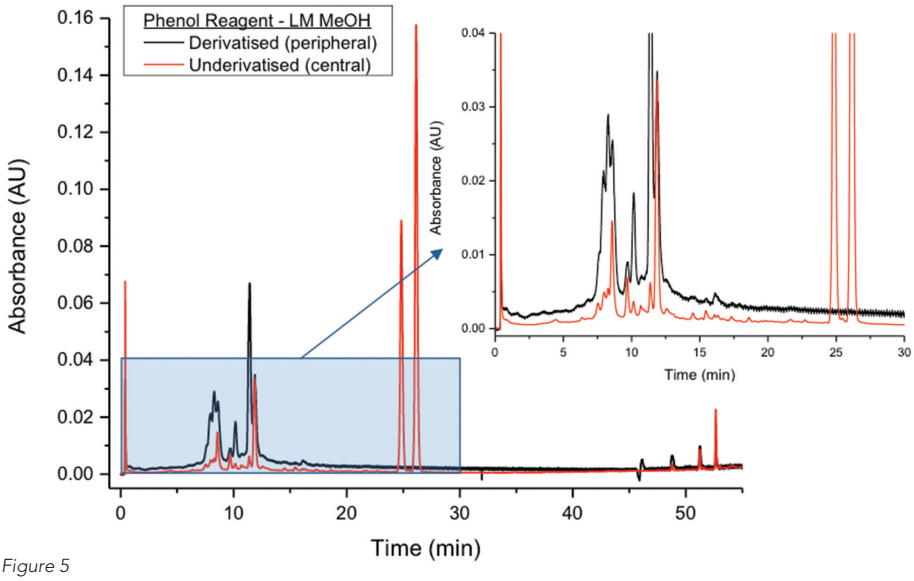

Figure 5