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High performance liquid chromatography with two simultaneous on-line antioxidant assays: Evaluation and comparison of espresso coffees

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ABSTRACT

The antioxidant profiles of various espresso coffees were established using HPLC with UV-absorbance detection and two rapid, simultaneous, on-line chemical assays that enabled the relative reactivity of sample components to be screened. The assays were based on (i) the colour change associated with reduction of the 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH•); and (ii) the emission of light (chemiluminescence) upon reaction with acidic potassium permanganate. Results from the two approaches were similar and reflected the complex array of antioxidant species present in the samples. However, some differences in selectivity were observed. Chromatograms generated with the chemiluminescence assay contained more peaks, which was ascribed to the greater sensitivity of the reagent towards minor, readily oxidisable sample components. The three coffee samples produced closely related profiles, signifying their fundamentally similar chemical compositions and origin. Nevertheless, the overall intensity and complexity of the samples in both UV absorption and antioxidant assay chromatograms were aligned with the manufacturers description of flavour intensity and character.

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1. Introduction

There is growing scientific evidence to suggest that many plant metabolites, such as ascorbic acid, tocopherols, carotenoids and phenolic compounds [1–3], participate in the cellular defense system against free radicals (i.e. exhibit *in vivo* antioxidant activity), offering numerous health benefits, such as antimutagenic, anticarcinogenic, and antiatherogenic effects [4–7]. Comparison of the antioxidant activity of foods (including the influence of their source and method of preparation), exploration of their key bioactive ingredients, and the search for new, potent antioxidants in foods and other plant-derived materials are therefore each of great interest in medicine, nutrition and food science.

Numerous model *in vitro* chemical assays have been developed to compare the relative reactivity of individual antioxidant compounds and/or assess the total antioxidant status of foods and biological fluids [8–15]. These systems include reactions with coloured 2,2-diphenyl-1-picrylhydrazyl (DPPH•) [16,17] or 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS•+) radicals [18,19], inhibition of peroxyl radical oxidation of fluorescent compounds [20,21], inhibition of the chemiluminescent oxidation of luminol [22,23], and many others. However, no single assay provides definitive results, due to factors such as the multiple mechanisms of antioxidant action, differences in the oxidant or free radical species used in each assay, and interferences specific to particular assays or classes of assay [8–15]. The use of multiple assays has been advocated to reconcile differences between antioxidant data [14], including an attempt to derive “a complete and dynamic picture of the ranking of food antioxidant capacity” [24].

Despite these issues, these model assays provide a convenient means to compare large numbers of samples and assess their potential for *in vivo* investigations. Over the past decade, several of these assays have been coupled to chromatographic separations to examine the relative antioxidant activity of individual components of complex plant-derived materials [25–27]. Compared to traditional bioassay-guided fractionation, this so-called *high resolution screening* offers rapid and cost-effective identification of key

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candidate molecules for structural characterisation and pharmacological/toxicological testing [26–28].

The on-line antioxidant assays reported to date have almost exclusively been based on DPPH• or ABTS•+ radical decolourisation, inhibition of luminol chemiluminescence, or electrochemical techniques [25]. All studies focused on a single on-line assay, with the exception of that described by Exarchou et al. [28], who used both DPPH• and ABTS•+ assays (after separate chromatographic runs) to examine the antioxidant profiles of several plant extracts. We recently proposed that the direct chemiluminescence reaction with acidic potassium permanganate could be exploited as a rapid on-line assay to screen for antioxidant compounds [29]. This reagent has previously been used for highly sensitive quantitative detection of phenols and related compounds after chromatographic separation [30], and to assess the total antioxidant status of wines, teas, and fruit juices using flow injection analysis methodology [29,31].

Coffee brews, due to their taste, fragrance and stimulating properties, are amongst the most popular beverages consumed throughout the world. It has been estimated that coffee-based drinks contribute 64% of the total antioxidant intake of the human diet [32] and the capacity of coffee to affect plasma redox homeostasis has been demonstrated [33]. Current literature suggests that the main compounds responsible for the antioxidant activity in roasted coffee are various phenols naturally present in green coffee beans (such as chlorogenic acids), and Maillard reaction products (melanoidins) formed in the roasting process [34–38]. Various off-line *in vitro* assays have be used to compare the total antioxidant activity of coffees of different origin, variety and brewing processes [36,38–41], examine fractions/compounds isolated from coffee [35,42,43], and as part of broader studies, comparing different plant extracts to identify rich sources of natural antioxidants [44] or examining the contribution of different foods to the total polyphenol/antioxidant consumption [45]. The application of on-line antioxidant assays to examine coffee is limited to two recent studies on the effects of roasting conditions, both of which combined reversed-phase (C18) chromatographic separation with the ABTS•+ radical scavenging assay [46,47]. The work described herein is the first use of an on-line DPPH• assay to provide a detailed antioxidant profile of coffee samples, which also serves as the first direct comparison of on-line DPPH• radical decolourisation and acidic potassium permanganate chemiluminescence assays.

2. Materials and methods

2.1. Chemicals and reagents

All mobile phases were prepared from HPLC-grade solvents purchased from Merck (Kilsyth, Victoria, Australia). All chemicals were commercially available. Potassium permanganate, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and sodium hexametaphosphate (crystals, +80 mesh) were purchased from Chem-Supply (Gillman, SA, Australia), Merck, and Sigma–Aldrich (Castle Hill, NSW, Australia), respectively. Milli-Q water (18.2 MΩ) was prepared in-house and filtered through a 0.2 μm filter.

2.1.1. Reagents

The DPPH• reagent (0.1 mM) was prepared in methanol. Solutions were prepared daily and protected from light. The acidic potassium permanganate reagent (5×10^{-4} M) was prepared by dissolution of potassium permanganate in a 1% (m/v) sodium hexametaphosphate solution and adjusted to pH 2.3 with sulfuric acid.

2.1.2. Samples

Sealed cartridges of Nestlé "Ristretto", "Gold", and "Decaffeinatto" espresso coffees were purchased. The manufacturer's description of these flavours is "subtle fruity full bodied" (intensity of 10), "sweet and biscuity" (intensity of 4) and "aroma of red fruit" (intensity of 2), respectively. The samples were brewed using a Nespresso coffee-maker, using the respective cartridges (5 g each). All coffees were prepared as 30 mL shots. Each shot was diluted 1:4 (with deionised water) prior to analysis. All samples were filtered through 0.45-μm pore filters prior to injection into the HPLC system.

2.2. Instrumentation

2.2.1. Chromatographic separation

All chromatographic experiments were conducted using a Waters 600E Multi Solvent Delivery LC System equipped with Waters 717 plus auto injector, two Waters 600E pumps, two Waters 2487 series UV/VIS detectors and two Waters 600E system controllers. Separations were performed on either a Kinetex 90 Å C18 (100 mm × 4.60 mm, 2.6 μm, P_d) column or a SphereClone 100 Å C18 (150 mm × 4.60 mm, 5 μm, P_d) column. Linear gradient conditions were employed on both columns, starting from an initial mobile phase composition of 100% water and running to a final mobile phase composition of 100% methanol, at a rate of 5% min^{-1}. The flow rate was 1 mL min^{-1} and the injection volumes were 10 μL. After UV-absorbance detection (280 nm), the eluate stream was split (50–50 ratio, controlled with a pressure regulator) at a T-piece for the two simultaneous on-line assays.

2.2.2. On-line DPPH• assay

One half of the eluate stream (0.5 mL min^{-1}) was combined with the DPPH• reagent (0.66 mL min^{-1}) at a T-piece. The combined stream entered a reaction coil (volume: 100 μL), which was submersed in a water bath maintained at 60 °C. Optimisation of this detection process is the focus of a separate study. Radical scavenging compounds were detected as a decrease in absorbance at 517 nm, using a Waters 2487 series UV/VIS absorbance detector.

2.2.3. On-line chemiluminescence assay

The other half of the eluate stream (0.5 mL min^{-1}) was merged with the acidic potassium permanganate reagent (1.85 mL min^{-1}) at a T-piece, immediately prior to entering a flow-through chemiluminescence detection cell, comprising a transparent coil of tubing mounted against the window of photomultiplier tube (Electron Tubes Model 9828SB, ETP, Ermington, NSW, Australia), in a light-tight housing [48]. The reagent was propelled to the T-piece using a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Balwyn, Victoria, Australia) with bridged PVC tubing (DKSH, Caboolture, Queensland, Australia). For comparison purposes, the time axes of the respective chromatograms were adjusted to account for the difference in volume between the column and the detectors for the DPPH• and chemiluminescence assays.

3. Results and discussion

Two critical aspects for high resolution screening are (i) maximising separation efficiency to isolate as many sample components as possible and (ii) minimising the time-scale of the assay (and thus the loss of resolution due to post-column band broadening), while maintaining sufficient sensitivity [25]. To these ends, we have coupled an efficient reversed-phase separation using a Kinetex C18 column with UV-absorbance detection and two rapid, simultaneous on-line chemical assays: DPPH• decolourisation and acidic potassium permanganate chemiluminescence. The proposed hyphenated system was used to examine the antioxidant profile of three espresso coffees.

3.1. Separation and detection conditions

High separation efficiency is crucial for the analysis of complex natural products. These types of samples contain multitudes of compounds, which often exceed the peak capacity of the separation space. This problem is compounded when multiple, sequential detectors are employed, or detection involves on-line chemical reactions, which can lead to significant post-column diffusive band broadening and loss of resolution. Therefore, to maximise detection of specific compounds, such as antioxidants, the chromatographic separation efficiency and the time-scale and degree of selectivity of each mode of detection must be considered.

We have combined a reversed-phase separation with UV-absorbance detection and two on-line chemical assays ($DPPH^{\bullet}$ decolourisation and acidic potassium permanganate chemiluminescence). The majority of previously reported on-line $DPPH^{\bullet}$ assays incorporated reaction coils constructed from 13 to 15 m of 0.25 mm i.d. tubing [25], which provided reactor volumes of over 600 μL, but significantly lower volumes have also been successfully used [49]. To provide sufficient reaction with minimal band broadening, we utilised a short reaction coil (100 μL volume) heated to 60 °C. The chemiluminescence detector consisted of tightly coiled transparent tubing (~40 cm of 0.8 mm i.d.), mounted against a photomultiplier tube. Although the total volume of this flow cell was approximately 200 μL, it should be noted that the width of the peaks are also dependent on the rate of the transient chemiluminescence response (i.e. the short-lived emission of light from a rapid chemiluminescent reaction may be complete before the reacting mixture exits the flow cell [50]). The aqueous-methanol gradient conditions selected for separation (described in Section 2.2) are compatible with both the $DPPH^{\bullet}$ decolourisation [25] and permanganate chemiluminescence [30] assays.

When combined with chromatographic separation, each of these three modes of detection provides a distinct perspective on the character of these highly complex sample matrices. Almost any solute with a suitable chromophore can be detected by absorption; 280 nm is most commonly used for the quantitative post-column detection of phenolic antioxidants in foods [51], but it is not specific to that functional group and provides no indication of reactivity. In contrast, the responses for the $DPPH^{\bullet}$ decolourisation and permanganate chemiluminescence assays are dependent on the reactivity of the compound towards the respective reagent (as well as the concentration of the compound) [10,52]. However, the mechanism of reaction and mode of detection are different [10,53]. The $DPPH^{\bullet}$ reagent is consumed by radical scavenging compounds to produce chromatograms comprising negative peaks from an ideally constant, high baseline signal (517 nm) [25]. The acidic potassium permanganate reagent provides highly sensitive detection of various phenols and other readily oxidisable compounds, based on the emission of light from the manganese(II) product of the reaction [53]. Unlike most other on-line assays used to assess the reactivity of antioxidant species [25], permanganate chemiluminescence produces positive signals on a low, stable baseline. These two assays are susceptible to very different interferences; examples include colour pigments of natural products that absorb light of the same wavelength as that used to measure $DPPH^{\bullet}$, and the remarkable sensitivity of the permanganate reagent towards certain phenolic alkaloids such as morphine and oripavine [54].

The importance of separation efficiency and the ramifications it has on detection is illustrated by the series of chromatograms in Fig. 1, which show separations achieved with a SphereClone C18 column (packed with conventional porous 5 μm particles) and a Kinetex C18 column (containing 'core–shell' 2.6 μm particles [55]), for the same sample under identical conditions. In each case, the upper trace represents UV-absorbance detection and the lower trace is the response for the $DPPH^{\bullet}$ assay. The difference between

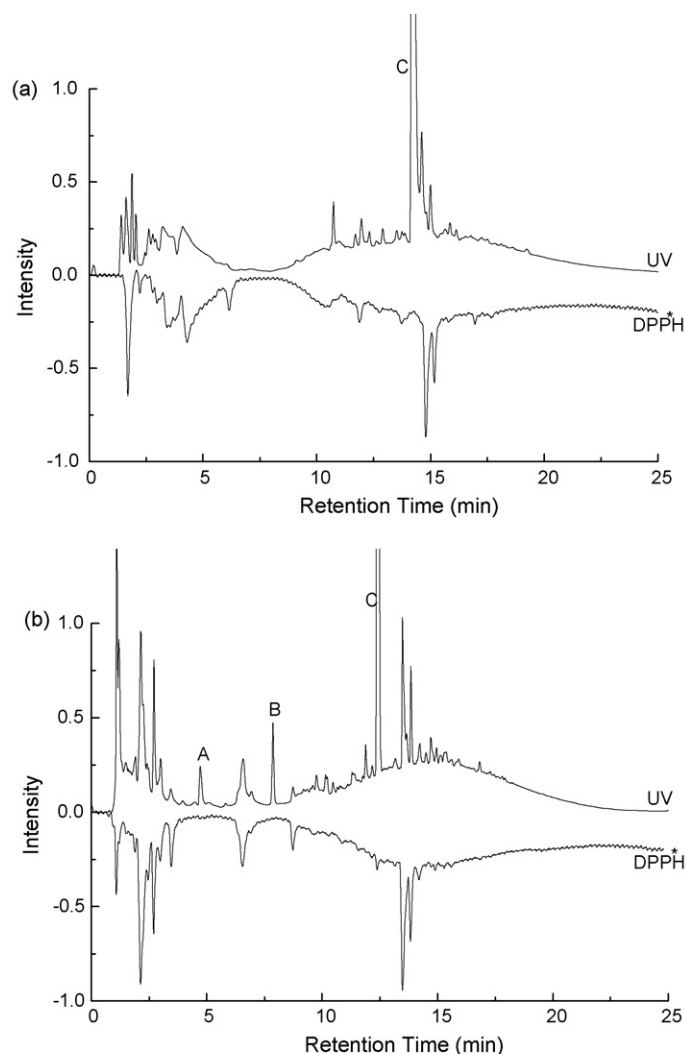

Fig. 1. Chromatograms for the Ristretto sample, separated on (a) SphereClone and (b) Kinetex columns. Response for UV-absorbance detection and $DPPH^{\bullet}$ assay shown.

the results obtained with the two columns was substantial. For example, the details of the $DPPH^{\bullet}$ response in the first 5 min of the analysis were lost in the complexity of the separation achieved on the SphereClone column, whereas the use of the Kinetex column allowed the direct association of many UV-absorbance peaks with $DPPH^{\bullet}$ detected bands. Of further interest was the discrimination of peaks that absorb ultraviolet light, but did not respond to the $DPPH^{\bullet}$ assay. Three examples, labelled as A, B and C (of which C is caffeine) in Fig. 1b, had virtually no $DPPH^{\bullet}$ response. While two of these bands were also observed in the separation on the SphereClone column, the third peak was less obvious. These were by no means exclusive examples of these types of peaks. Greater separation could have been obtained on the SphereClone column by decreasing the gradient rate, but at the detriment of analysis time and band broadening. Better separation was achieved on the shorter Kinetex column, using the same gradient rate and overall analysis time. All subsequent data reported in this work were obtained from separations using the Kinetex column.

3.2. Comparison of espresso coffees

The chromatograms obtained with UV-absorbance detection are shown in Fig. 2. Overall, the three coffees showed very similar fundamental chemical composition, within the limitations of the

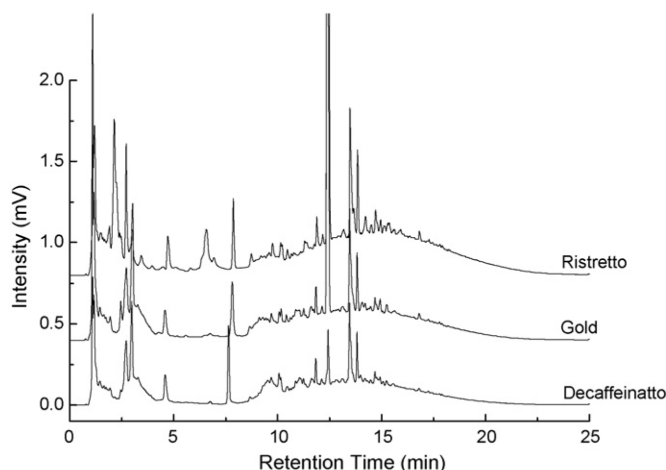

Fig. 2. Chromatograms for separation on Kinetex column and UV-absorbance detection, of Ristretto, Gold and Decaffeinatto café espresso samples.

information that can be derived from a unidimensional separation of this highly complex matrix. In general, the sample complexity of the "Ristretto" coffee was greater than those of both the "Decaffeinato" and the "Gold" espressos, which is consistent with the product description. The chromatographic profiles of the "Gold" and "Decaffeinatto" espressos were almost perfectly overlaid; thus it is tempting to suggest that the decaffeinated coffee was derived from similar beans to those used in the preparation of the "Gold" espresso.

The chromatograms obtained with the DPPH• decolourisation and permanganate chemiluminescence assays indicated that all three coffees contained a substantial number of antioxidant-type compounds. The chromatograms in Fig. 3, for example, compare the results of the three methods of detection for the Ristretto sample. Table 1 lists the most significant peaks in all three modes of detection, in order of retention time. Detection was rated with a score of 0–3, with 0 indicating no peak detected and 3 indicating an important peak. This relative score does not give any information regarding the absolute nor even the relative concentration of each component. If two modes of detection scored a 0 response while the third detector scored a significant response, the rating was 3 by default. If two detectors were equally sensitive and more so than the third, they both scored a value of 3. Of the 21 peaks listed in Table 1, 19 components were observed with UV-absorbance (280 nm) detection, with 70% yielding a strong response. Only 13 of the components responded to the DPPH• assay, with 28% showing a strong response. In the chemiluminescence assay, 16 components were seen, with 52% showing a strong response, indicating that, compared to DPPH•, this reagent is sensitive towards a wider range of oxidisable sample components. This clearly illustrates the advantage of employing multiple modes of detection when searching for bioactive species in complex media. Each mode was able to discriminate between sample components depending on certain characteristics. In particular, it was interesting to examine the degree of discrimination between chemiluminescence and DPPH• assays, which revealed the different behaviour of vari-

Table 1
Key peaks in the chromatograms for the Ristretto coffee sample obtained using UV-absorbance, DPPH• and chemiluminescence modes of detection.

Peak	Retention time (min)	UV	DPPH•	CL
1	1.09	3	1	2
2	1.18	3	1	2
3	2.13	3	3	3
4	2.50	1	1	3
5	2.71	3	2	3
6	3.5	1	3	2
7	4.71	3	0	2
8	6.57	3	3	3
9	7.40	0	0	3
10	7.87	3	0	0
11	8.73	1	3	3
12	10.2	1	0	3
13	10.45	3	0	0
14	11.87	3	0	0
15	12.44	3	1	1
16	13.50	3	3	3
17	13.85	3	3	3
18	14.23	3	2	3
19	14.52	3	1	0
20	14.69	3	0	0
21	15.65	0	0	3

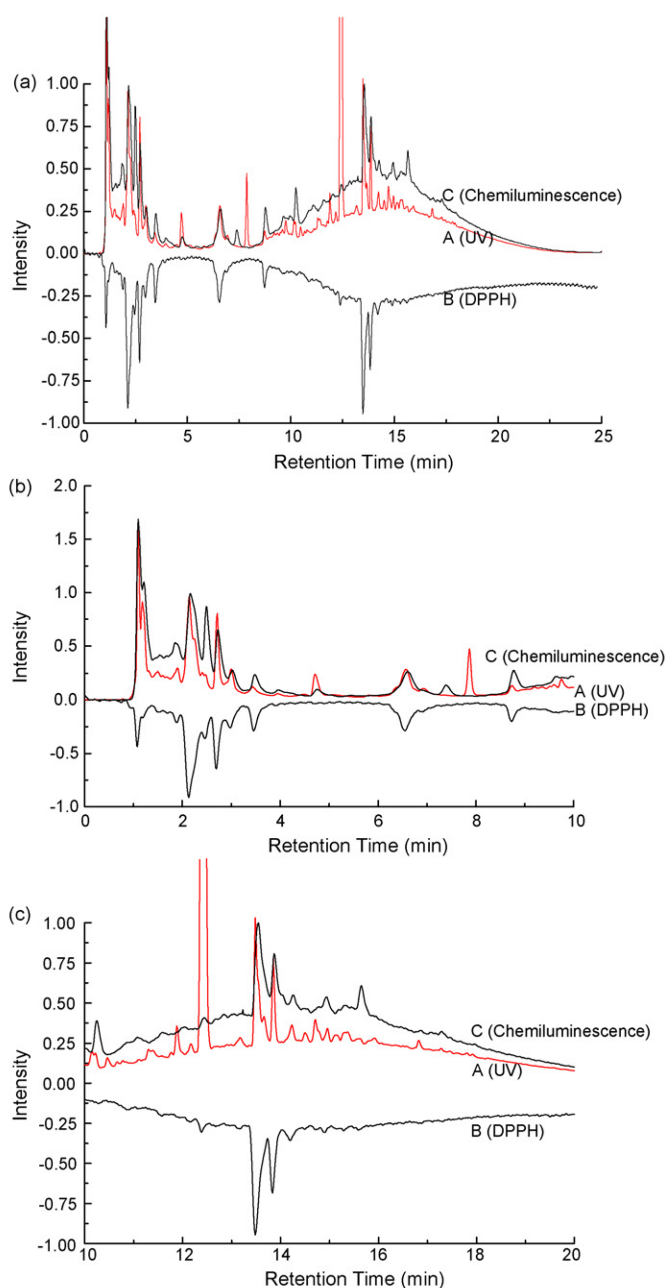

Fig. 3. (a) Chromatograms for separation of Ristretto coffee with (A) UV-absorbance detection, (B) DPPH• decolourisation assay, and (C) acidic potassium permanganate assay. (b) and (c): As above, with close up view of 0–10 min and 10–20 min, respectively.

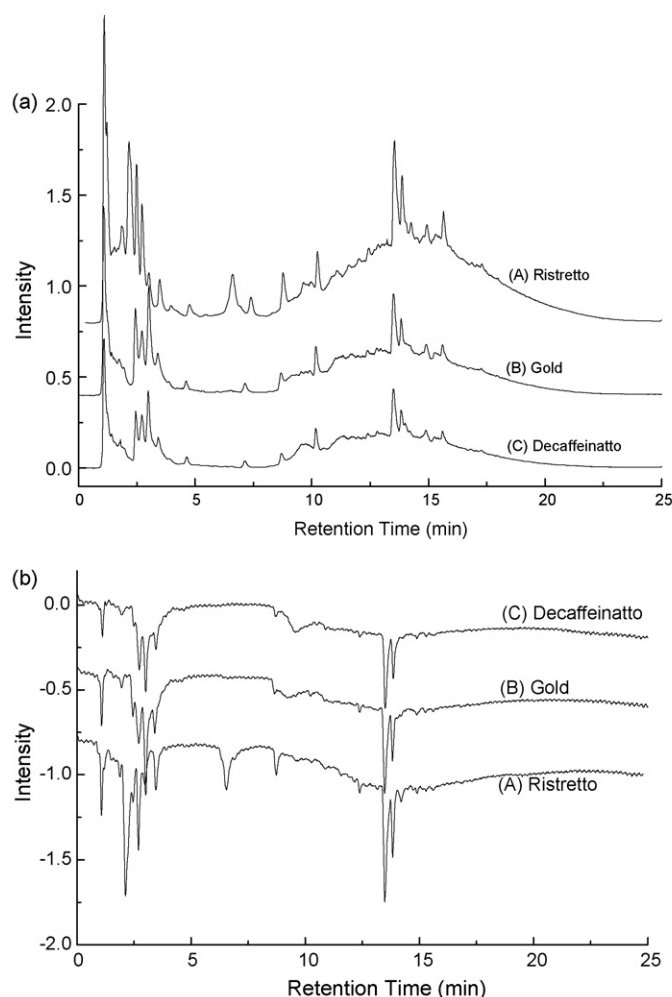

Fig. 4. Chromatograms for Ristretto, Gold and Decaffeinatto samples: (a) acidic potassium permanganate assay and (b) DPPH• decolourisation assay.

ous oxidisable compounds contained in the coffee samples. Hence, using multiple modes of detection may aid in not only identifying antioxidant species, but also understanding their mode of action.

Comparison of the three coffee samples based on permanganate chemiluminescence or DPPH• decolourisation assays (Fig. 4) shows a degree of similarity akin to that observed with UV-absorbance detection. Interestingly, the overall intensity of the response for both assays was aligned with the flavour 'intensity' scale provided by the manufacturer (2, 4 and 10, for Decaffeinatto, Gold and Ristretto, respectively). In agreement with previous comparisons of the total antioxidant activities of coffees (with and without decaffeination) using off-line *in vitro* assays [39], and examination of the antioxidant profile of coffees using HPLC with an on-line ABTS•+ assay [47], caffeine did not exhibit antioxidant activity. However, caffeine has previously been shown to be an effective inhibitor of lipid peroxidation (*in vitro*) induced by hydroxyl (HO•) and peroxyl (LOO•) radicals and singlet oxygen ($^1O_2$) [56]. Moreover, Brezova et al. recently noted that although inert to ABTS•+ and DPPH•, caffeine is effective in scavenging HO• radicals [38]. This demonstrates an important limitation of off-line and on-line *in vitro* assays for antioxidant activity, where some compounds that do not respond to particular assays may still exhibit significant activity under other conditions. Nevertheless, the overall antioxidant profile of the "Decaffeinato" coffee sample was similar to those of the "Gold" and "Ristretto" samples – rich in compounds that responded to both on-line assays – and it is therefore likely that decaffeinated coffees have similar positive effects on human health.

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