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The analysis of café espresso using two-dimensional reversed phase–reversed phase high performance liquid chromatography with UV-absorbance and chemiluminescence detection

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ABSTRACT

In this study, an activity based screening technique combining two-dimensional liquid chromatography (2DHPLC) with UV-absorbance and chemiluminescence detection was applied to study "Ristretto", "Decaffeinatto" and "Volluto" espresso coffees. This technique, which coupled the separation power of 2DHPLC with the sensitivity and selectivity of the chemiluminescence detection, offers great potential for screening complex samples for antioxidant compounds. Detailed information regarding the complexity of the sample, and the variation between these three coffees could be obtained using this multidimensional-hyphenated method of analysis.

1. Introduction

Antioxidants have received growing interest as active agents in various natural products offering benefits to human health [1,2]. Sources of natural antioxidants range from marine sponges [3] to microbes [4] and plants [5] and they are considered by many to be revolutionising foods, medicines, and cosmetics [6–9] serving as either a substitute for synthetic compounds or as active ingredients for health fortifying purposes [10]. Thus finding new "unconventional sources" of functional molecules, and in particular antioxidants, could lead to new and important discoveries. Tulp et al. suggested that foods and beverages, primarily not known for their medicinal properties, could potentially be the next valuable source of natural compounds that require the attention of the scientific community [11].

Roasted and green coffee beans have extremely complex chemical compositions, containing large numbers of components with a wide range of properties and sizes; some of which (e.g. chlorogenic acids and related phenolic compounds, and Maillard reaction products (melanoidins)) [12–14] are strong antioxidants with beneficial physiological properties for human health [15]. The chemical profile becomes even more complex based on the geographical origin, roasting degree, and the type of coffee beans [16,17]. Coffee is an important source of natural antioxidants, because it is consumed worldwide, throughout almost every culture. Accordingly, analytical techniques that provide reliable separation and analysis of antioxidants from the complex coffee matrix could be of great importance.

Recently, much effort has been directed towards accelerating the screening and evaluation of antioxidant content in foods and plants. So far, modification of traditional batch type antioxidant assays into so-called high resolution screening techniques that combine detection with separation are showing the greatest promise to rapidly discover key antioxidant compounds [18–21]. These on-line screening assays, which substantially reduce discovery time and cost [22], are important hyphenated methods of analysis, but once the peak capacity of the separation process is exceeded, the ability of the hyphenated detector to provide unequivocal information about specific compounds decreases as the complexity of the sample increases. Hence, separation (according to information, i.e. the hyphenated mode of detection) must be transposed to the physical separation, that is, chromatographically. In recent times there has been a drive towards more powerful separations that incorporate multiple selectivity steps (i.e.

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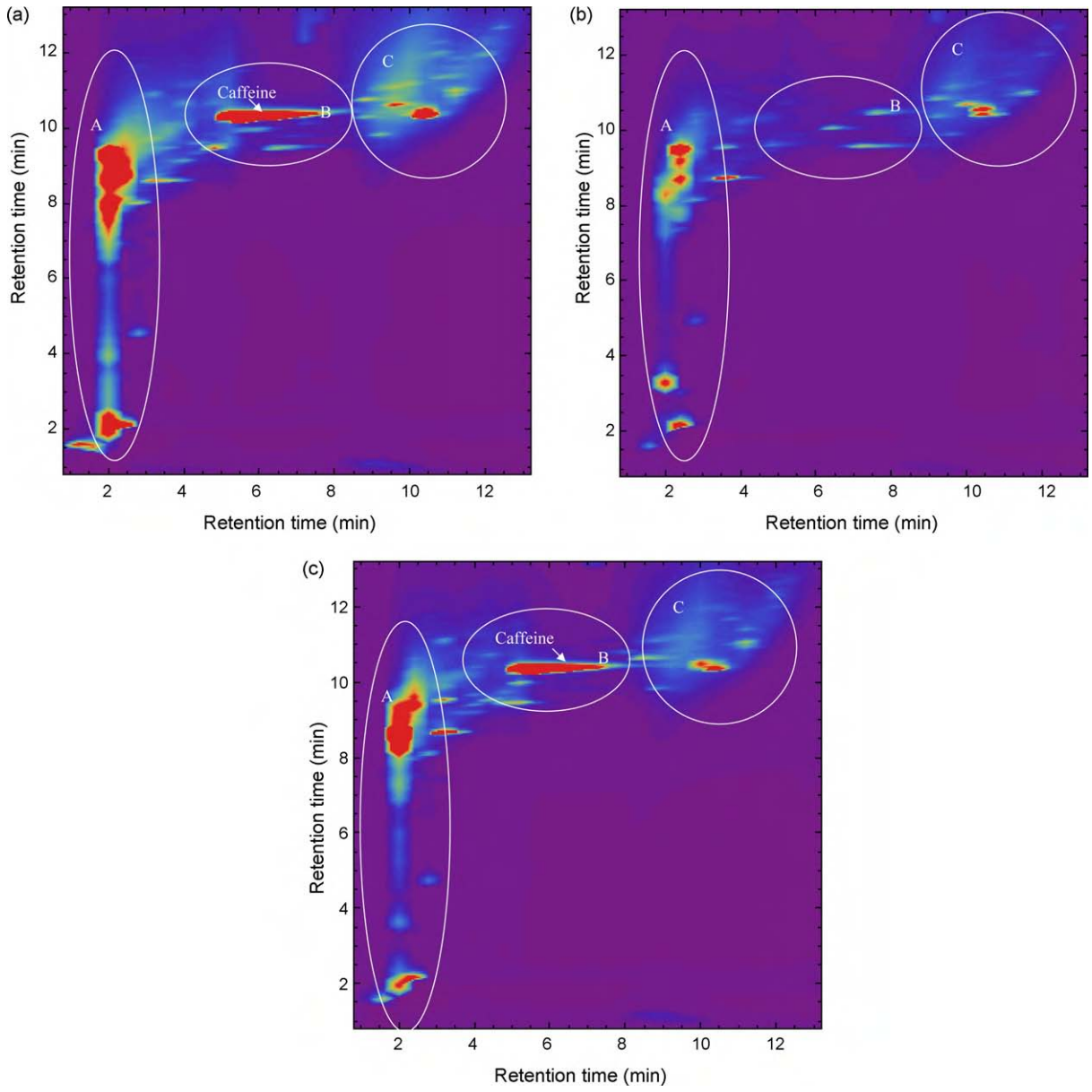

Fig. 1. Two-dimensional separations of (a) Ristretto, (b) Decaffeinato and (c) Volluto café espresso. First dimension Cyano and second dimension C18 phases. In both dimensions mobile phase was aqueous methanol, going from 100% water to 100% methanol. All conditions identical for each phase system.

multidimensional HPLC, more often referred to as two-dimensional HPLC (2DHPLC)) [23,24].

The combination of the powerful separation process with that of antioxidant detection should enable the rapid identification and subsequent targeted extraction of bioactive compounds from complex sample matrices [25]. Here we demonstrate the application of 2DHPLC using chemiluminescence detection in the search for antioxidants in espresso coffees. A comparative study was undertaken that tested three types of café espresso coffees, which included a decaffeinated variety. The two-dimensional chromatographic system consisted of a cyano stationary phase and a C18 stationary phase, both employing water/methanol gradient elution mobile phases. The chemiluminescence detection involved a post-column reaction with acidic potassium permanganate. This reagent provides highly sensitive detection of polyphenols and various other readily oxidisable compounds [26,27] and has previously been utilised to establish the total antioxidant capacity of teas and fruit juices (using flow-injection analysis methodology) [28] and to explore the relative reactivity of individual sample components after chromatographic separation [25,28].

2. Experimental

2.1. Chemicals and reagents

All mobile phases were prepared from HPLC grade solvents and were purchased from Lomb Scientific (Tarren Point, NSW, Australia). Sodium hexametaphosphate (crystals, +80 mesh) was purchased from Merck (Kilsyth, Victoria, Australia). Potassium permanganate was purchased from Chem-Supply (Gillman, SA, Australia). Milli-Q water (18.2 MΩ) was obtained in-house and filtered through a 0.2 μm filter.

2.2. Reagent preparation

The chemiluminescence reagent was prepared by dissolution of potassium permanganate (5×10^{-4} M) in a 1% (m/v) sodium hex-

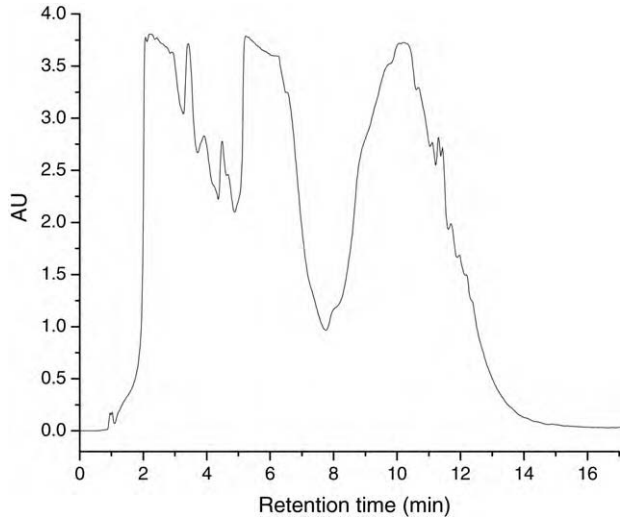

Fig. 2. One-dimensional separation of Ristretto café espresso on Cyano column. Mobile phase was aqueous methanol, going from 100% water to 100% methanol.

ametaphosphate solution, and was adjusted to pH 2.3 with sulfuric acid.

2.3. Sample preparation

The cartridges of "Ristretto", "Decaffeinatto" and "Volluto" espresso coffees were obtained from the local market (Nespresso Australia, North Sydney, NSW, Australia). The coffee brews were prepared as a 30 mL shot using a "Nespresso" coffee-maker, using the respective cartridges (5 g each). All samples prior to injection into the HPLC system were filtered through 0.45-μm pore filter prior to analysis.

2.4. Instrumentation

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. Chromatographic separations were performed on a Phenomenex Luna 100 Å CN (150 mm × 4.60 mm × 5 μm particle diameter (Pd)) in the first dimension, and SphereClone 100 Å C18 (150 mm × 4.60 mm × 5 μm Pd) column in the second dimension (Phenomenex, Lane Cove, NSW, Australia). The same linear gradient conditions were employed on both columns, starting from an initial mobile phase composition of 100% water, running to a final mobile phase composition of 100% methanol at a rate of 10% per min. These separation conditions were obtained following extensive optimization [29]. The flow rate was 1 mL/min and injection volumes in the first dimension were 100 μL. The chromatographic interface between the first and second dimensions consisted of electronically controlled two-position six-port valves fitted with micro-electric two-position valve actuators that allowed alternate sampling of the elute from the first dimension into the second dimension. Following UV-absorbance detection (280 nm), the HPLC column eluent was sent to a chemiluminescence detector comprising of a T-piece and transparent reaction coil mounted against the window of a photomultiplier tube (Electron Tubes Model 9828SB, ETP, Ermington, NSW, Australia), in a light-tight housing [30]. The chemiluminescence reagent was propelled to the T-piece using a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Balwyn, Victoria, Australia).

Table 1
Number of peaks detected for each café espresso flavour for both UV-absorbance and chemiluminescence detection.

Coffee flavour	UV detected peaks	CL detected peaks
Ristretto	138	65
Volluto	88	56
Decaffeinato	68	44

2.5. Chromatographic separation

2.5.1. Comprehensive "heart-cutting" 2D separations

The comprehensive heart-cutting 2DHPLC analysis of each coffee brew was undertaken such that the eluent flow stream from the first dimension (200 μL) was transferred using a heart-cutting process into the second dimension. The second dimension separation was completed, and then a second sample was injected into the first dimension. The heart-cutting process was repeated, each time sampling every second 200 μL portion of eluent from the first dimension. In total, the first dimension was sampled 34 times resulting in a total analysis time of 21 h. Data plotting and calculation of peak recognition were undertaken using *Mathematica 7* [31].

3. Results and discussion

Fig. 1 illustrates the two-dimensional surface plot (UV-absorbance detection) of the chromatographic separation of (a)

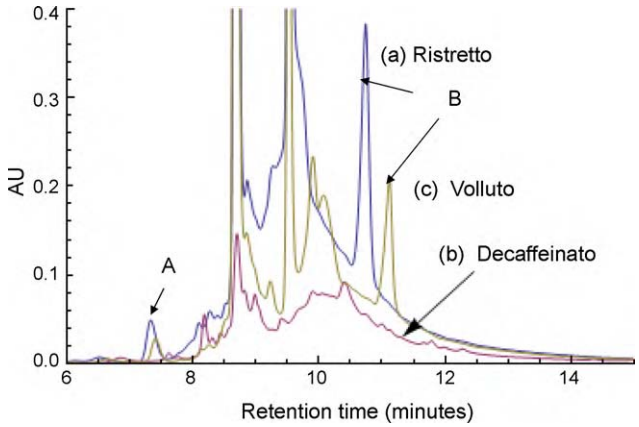

Fig. 3. Heart-cut segment separation of (a) Ristretto, (b) Decaffeinato and (c) Volluto café espresso on C18 column at 3.2 min.

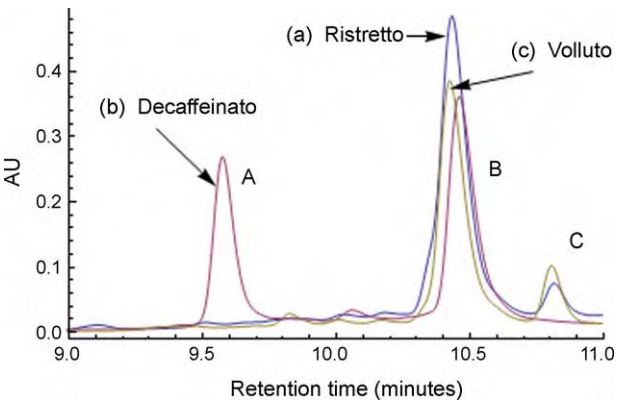

Fig. 4. Overlay of the separations of (a) Ristretto, (b) Decaffeinatto and (c) Volluto café espresso on C18 column heart cut at 7.6 min. Peaks A and B in (b) mark peaks in this region.

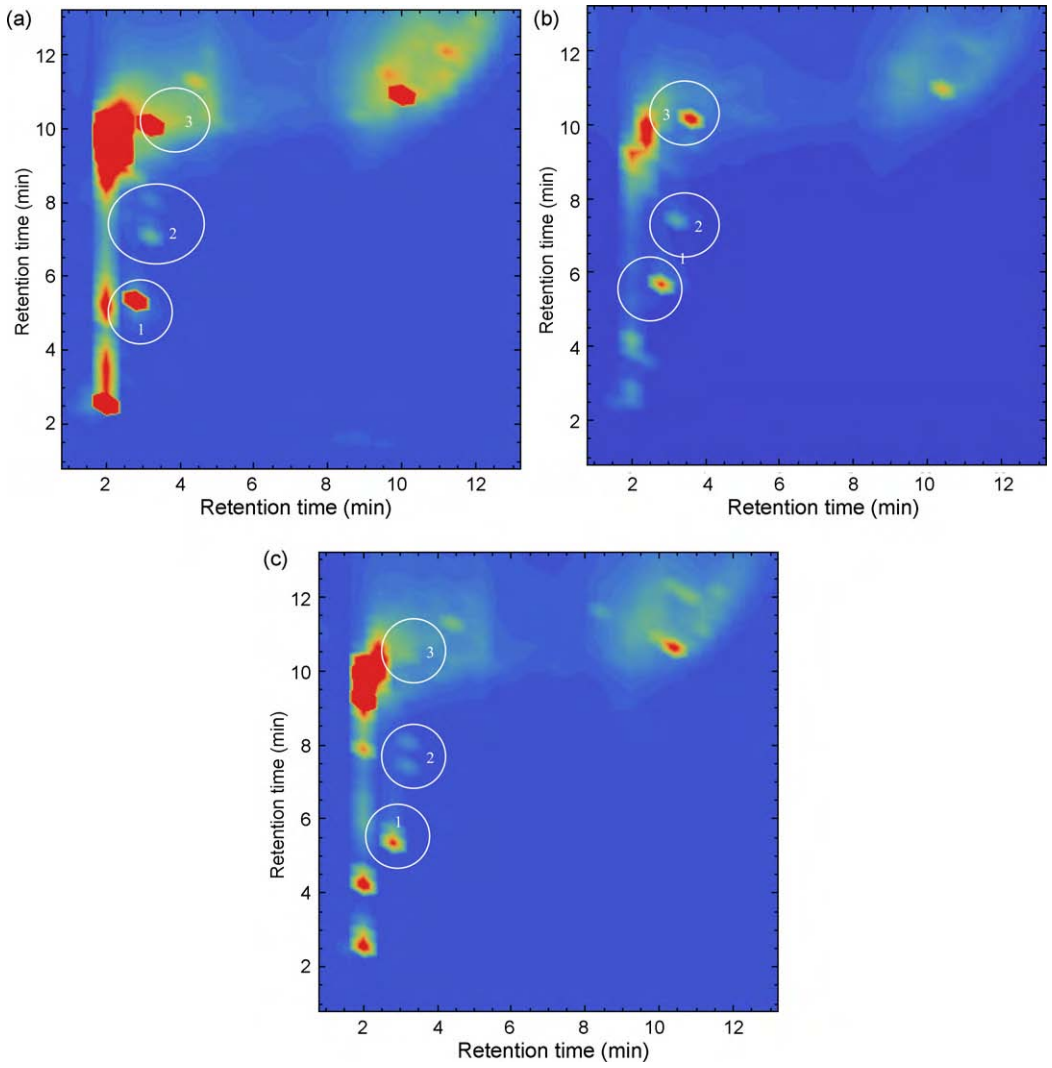

Fig. 5. Chemiluminescence detection plots of (a) Ristretto, (b) Decaffeinato and (c) Volluto café espressos.

Ristretto, (b) Decaffeinato, and (c) Volluto coffee brews. Each of these three samples produced very similar chromatographic elution profiles across the two-dimensional domain, which was not unexpected given the similarity of their unidimensional separations, depicted in previous works [32]. A noteworthy difference between each sample of coffee was the intensity of the elution profiles, which decreased in accord to the label claim associated with the 'strength' of the brew.

Interestingly, given the complexity of the sample, retention displacement was effectively reduced to three primary zones within the two-dimensional separation plane, listed as region A, region B and region C. The two-dimensional separation plane bound by the Cyano and C18 phases displayed significant differences in retention behaviour within specific regions of the separation space. For example, for the compounds in region A (mainly low molecular weight hydroxylated and phenol compounds, carboxylic acids [29]), which were hardly separated on the first dimension (cyano column), but had profound retention and subsequent separation in the second dimension (C18), the retention correlation was almost zero, or in other words, these two dimensions were orthogonal for the compounds separated here. The compounds in region B were selectively separated on the cyano phase, with less separation on the C18 phase, and in this region correlation between both dimensions increased, yet the correlation coefficient was only ~0.5, indicating significant differences were still apparent in the retention mechanisms between the dimensions. Amongst these compounds in region B was caffeine, which dominated the separation plane, being present as the most abundant species within the entire sample. Compounds in region C where strongly retained on both phases, and hence correlation in this region of the separation increased to a correlation coefficient of approximately 0.8 [29].

In comparison to the 2D separations depicted in Fig. 1, limited information about the sample was obtained from either respective 1D separations, as shown by the example of the separation of the Ristretto coffee (Fig. 2) obtained on the cyano column under the conditions employed for the 2D separation. This separation shows a continuum of sample, partly due to the complexity of the sample and the fact that the peak capacity was exceeded, and partly because the column was overloaded with sample. Nevertheless even at lower sample loads the sample contains too many components to yield separation in a unidimensional sense [32].

Fig. 3 illustrates the separation on the C18 column (unidimensional 2nd dimension separations) of heart cut fractions from the first dimension of the weakly retained species on the cyano column for each of the three coffees: (a) Ristretto; (b) Decaffeinatto; (c) Volluto. The heart cut sections were at 3.2 min from the Cyano dimension. These separations highlight the significant change in selectivity (practically orthogonal) between each dimension [29] and at the same time illustrate some of the differences between each of these coffee brews.

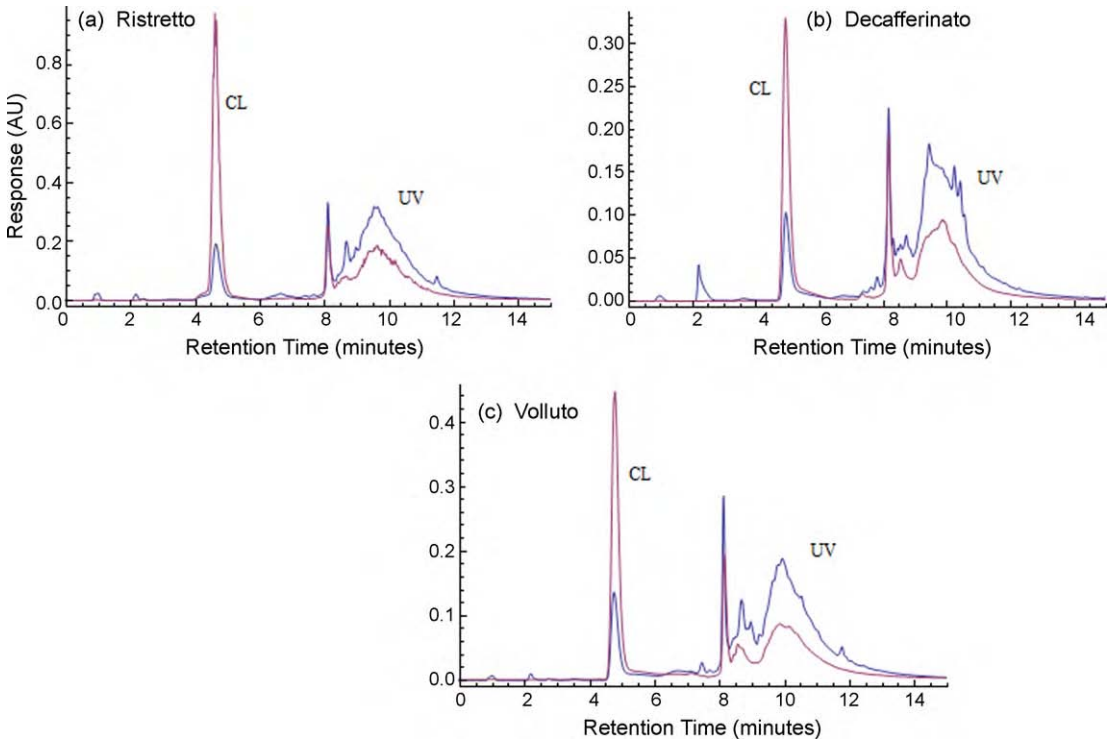

Fig. 6. UV-absorbance and chemiluminescence detection response of heart-cut fractions of (a) Ristretto, (b) Decaffeinato and (c) Volluto café espresso at 2.8 min. Blue and red lines represent UV-absorbance and chemiluminescence response, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

While each of the three espressos showed similar bulk behaviour, there were, however, subtle differences in their chemical composition, as well as the notable difference in the lack of caffeine in the Decaffeinato sample (Fig. 1(b) compared to Fig. 1(a) and (c)). What is surprising is the specificity associated with the removal of caffeine in the decaffeinated sample. Examination of the compounds that neighbour the caffeine peak across both dimensions reveals that there was limited interference to these compounds. Although, in the Decaffeinato sample, a new peak is apparent that neighbours the caffeine band region. The new compound is not present in either the Ristretto or Volluto samples, meanwhile both Ristretto and Volluto have a single compound eluting at 10.8 min that is absent from the Decaffeinato sample. Fig. 4 illustrates the unidimensional heart cut slice separated on the C18 column derived from the 7.6 min region on the cyano dimension, for the Ristretto, Decaffeinato and Volluto samples, respectively, illustrating the subtle difference associated with this region of the sample. These differences could only be visualised through high peak capacity separations.

It is worth further detailing some of the differences that were apparent in Fig. 3 between each of the three coffees for the heart cut section at 3.2 min of the cyano phase. In this particular heart cut region all three coffees were different. In particular the Decaffeinato coffee was the least complex, with a lower total intensity. Of note, is the presence of two bands labelled as 'A' and 'B' that were present in the Ristretto and Volluto coffees, respectively. Neither of these bands was present in the Decaffeinato coffee. Thus demonstrating the fingerprinting potential of this technique, especially for the description of sensory attributes of food samples.

Fig. 5 illustrates the surface plots of two-dimensional separations for each of the three coffee brews (Ristretto, Decaffeinato and Volluto, respectively) using the acidic potassium permanganate chemiluminescence detection. In contrast to UV-absorbance detection, an intense emission with this chemiluminescence reagent is indicative of a reducing agent (antioxidant) with relatively high concentration and/or reactivity [33]. Substantial differences in the UV-absorbance and chemiluminescence detection were apparent. Most notable was the complete absence of the caffeine band in the chemiluminescence profile of both the caffeinated samples. Furthermore, the new band labelled as 'A' in Fig. 4(b) was not responsive to this chemiluminescence reagent.

More importantly, the chemiluminescence detection enabled visualisation of compounds with 'apparent high antioxidant activity' that were almost entirely absent in the UV-absorbance detection mode, thus providing complementary information about the sample matrix [28,32]. The chemiluminescence detection primarily exhibited sensitivity towards the range of compounds present in regions A and C (see Fig. 1) of the sample, and was not responsive to compounds in region B, suggesting that most compounds in that region are not significant antioxidants. In contrast, compounds highlighted as 1 in Fig. 5 responded weakly in terms of UV-absorbance, but exhibited intense chemiluminescence , indicating that these compounds may possess strong antioxidant activity. This is consistent with out observations made during the optimization of the separation whereby we found that the compounds eluting here were mainly carboxylic acids. Also of interest is the elution of the band labelled as 2 in Fig. 5, which was only visible in the chemiluminescence mode of detection.

Another region of distinct difference in the detection responses is that of region 3 in Fig. 5, where strong chemiluminescence is observed, but only weak UV absorption. These differences are more clearly shown in the series of UV-absorbance and chemiluminescence detection responses separated on the C18 dimensions from heart cut sections at 3.2 min on the cyano column (Fig. 6). Overall the benefit of such combinations of different modes of detections, complementary to each other, is substantial and especially important in bio-assay based screening analysis. Furthermore, the ability

to detect the potential bioactive compound, and the enhanced separation power of the 2D system would enable the rapid isolation of these targeted components from within this complex sample matrix.

Overall there are numerous subtle differences between each of these three coffee brews, depicted in both the UV-absorbance and chemiluminescence detection modes. However, these differences are too complex to note individually; rather, it is easy to simply state the difference in the number of components that were separated and subsequently detected. In part these differences may arise because of the strength profile in each coffee brew. The number of detected peaks for each sample (Table 1) was established using a peak picking algorithm, discussed in prior works [31]. The least number of peaks, in both modes of detection, was observed in the Decaffeinatto sample, which was described by the manufacturer as the 'weakest' of the coffee brews. The strongest of the coffee brews, Ristretto, had the most number of peaks visible in the chromatograms obtained using UV-absorbance detection, but the Volluto sample had more peaks present when chemiluminescence detection was used.

Overall, the data obtained through the current combination of two-dimensional separations with both UV-absorbance and acidic potassium permanganate chemiluminescence detection offers relevant and comprehensive information for chemical matrix characterisation and could serve as a fingerprint for the particular sample description. Furthermore, the technique can be used to target the isolation of key antioxidants from these complex matrices with a relative degree of simplicity. These types of analyses also have the potential to generate simultaneously valuable data on the bio-markers and become of special importance for designing complete food "beneficial" compositional tables required for epidemiological research. Future studies in this area will involve the identification of key antioxidant species, and the investigation into whether or not 2DHPLC, with and without chemiluminescence detection may be utilized for the purpose of identifying the origin of the coffee beans.

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