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Screening for antioxidants in complex matrices using high performance liquid chromatography with acidic potassium permanganate chemiluminescence detection

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Thyme

ABSTRACT

The use of high performance liquid chromatography with acidic potassium permanganate chemiluminescence detection to screen for antioxidants in complex plant-derived samples was evaluated in comparison with two conventional post-column radical scavenging assays (2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•+)). In this approach, acidic potassium permanganate can react with readily oxidisable compounds (potential antioxidants), post-column, to produce chemiluminescence. Using flow injection analysis, experimental parameters that afforded the most suitable permanganate chemiluminescence signal for a range of known antioxidants were studied in a univariate approach. Optimum conditions were found to be: 1×10^{-3} M potassium permanganate solution containing 1% (w/v) sodium polyphosphates adjusted to pH 2 with sulphuric acid, delivered at a flow rate of 2.5 mL min^{-1} per line. Further investigations showed some differences in detection selectivity between HPLC with the optimised post-column permanganate chemiluminescence detection and DPPH• and ABTS•+ assays towards antioxidant standards. However, permanganate chemiluminescence detection was more sensitive. Moreover, screening for antioxidants in green tea, cranberry juice and thyme using potassium permanganate chemiluminescence offers several advantages over the traditional DPPH• and ABTS•+ assays, such as faster reagent preparation and superior stability; simpler post-column reaction manifold; and greater compatibility with fast chromatographic separations using monolithic columns.

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1. Introduction

The ability of natural plant-derived antioxidants to protect against oxidative damage has been comprehensively studied from various perspectives [1–8]. The primary focus, however, has been on their potential to assist endogenous antioxidants in preventing free-radical induced oxidative stress within the human body, implicated in a growing number of pathophysiological conditions (adriamycin cardiomyopathy, aging, diabetes, cancer, myocardial infarction, Parkinson's disease, stroke, etc.) [1–4,6]. Another well documented application is their use in foodstuffs to replace possibly toxic synthetic antioxidants such as butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ); added to products to prolong their shelf life through prevention of lipid peroxidation [5,7,8]. Consequently, the discovery and assessment of antioxidants in complex matrices is of the upmost importance to researchers in a diverse range of fields, such as nutrition, medicine, food and agricultural science, and drug discovery [1–8].

Many off-line, batch style methodologies have been developed for the determination of antioxidants [9–12]. These assays are routinely employed to measure the total antioxidant capacity of plant and food matrices, but owing to sample complexity they generally prove to be insufficient for the assessment of individual antioxidants [9–12]. Instead, this task has traditionally been achieved using bioassay-guided fractionation; reportedly an expensive, labour-intensive and time-consuming process that may lead to the loss of a compound's activity [13–17]. However, as an alternative, researchers have recently modified several

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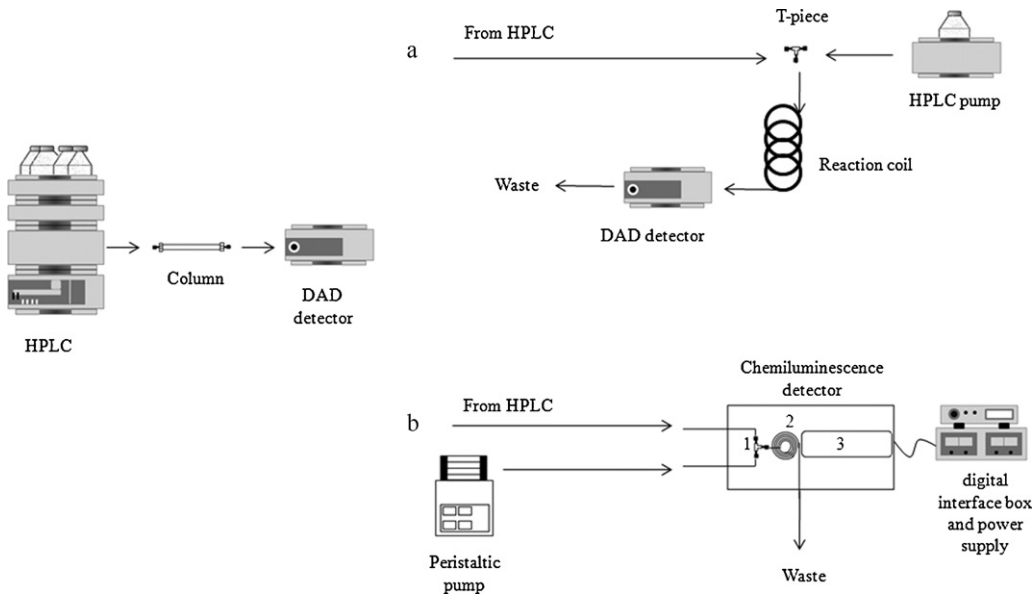

Fig. 1. Instrument setup for HPLC with post-column reactions: (a) DPPH• and ABTS•+; (b) acidic potassium permanganate chemiluminescence. (1) T-piece; (2) transparent PTFE-PFA tubing reaction coil; (3) PMT.

batch analysis techniques, developing continuous-flow antioxidant detectors that can be coupled to high performance liquid chromatography (HPLC) systems [13,14]. Over the last decade, these post-column antioxidant activity-based detectors have appeared in over 40 publications and are the subject of reviews by Niederländer et al. [13] and Shi et al. [14]. The majority of applications employ free radical decolouration reactions, based upon either the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) or the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•+) [13,14]. Briefly, this approach involves merging a solution of the DPPH• or ABTS•+ reagent with the post-column HPLC eluate at a T-piece (using a second HPLC pump for the reagent line). The resulting stream is then passed through a reaction coil of adequate length and internal diameter to provide sufficient time for the reagent to react with any radical scavenging (i.e. antioxidant) species present, before entering a spectrophotometric detector (Fig. 1a). Thus, radical scavenging compounds are detected as negative peaks as reduction of the DPPH• or ABTS•+ reagent leads to a significant shift in their UV–vis absorption spectrum [13,14].

Another less common type of on-line antioxidant assay employs indirect luminol chemiluminescence detection [13,14,18–20]. Here, syringe pumps are used to merge streams of luminol and an oxidant (typically hydrogen peroxide) at a T-piece, to generate chemiluminescence. This mixture is then combined with the HPLC eluate at a second T-piece and the resulting solution passed through a reaction coil and to a photo-detector (Fig. S1; see Electronic Supplementary Information). As with the radical decolouration assays described above, antioxidants are detected as negative peaks, as they inhibit the reaction between luminol and oxidants causing quenching of the background chemiluminescence signal [13,14,18–20].

High performance liquid chromatography with post-column acidic potassium permanganate chemiluminescence detection has been used for the determination of many oxidisable compounds [21,22], and is an attractive option for the screening of potential antioxidants in complex matrices [23–25]. In this protocol a peristaltic pump is employed to merge acidic potassium permanganate with the HPLC eluant at a T-piece directly connected to a coiled flow cell that is mounted flush against a photomultiplier tube (PMT). All equipment is housed inside a light tight box (Fig. 1b). Here, acidic potassium permanganate can react with potential antioxidants to produce light [23–25], which emanates from an electronically excited manganese(II) species [26]. Unlike the other post-column antioxidant assays, compounds are detected as positive peaks from a stable background rather than negative signals from inhibition of a high background. The term 'antioxidant' in the context of this methodology is defined as a readily oxidisable compound, meaning not all reductants that produce light with this reagent [21,22] may be considered as dietary or primary antioxidants as outlined by Halliwell and Whiteman [27] and Huang et al. [9]. Nevertheless, many known antioxidants (e.g. gallic acid, quercetin, resveratrol and rosmarinic acid [23,28]) have been shown to result in a relatively intense emission.

Using FIA methodology, we recently developed a procedure to measure the total antioxidant capacities of numerous plant-derived beverages based on acidic potassium permanganate chemiluminescence, which showed good agreement with conventional DPPH• and ABTS•+ batch assays [29]. This study included a demonstration of HPLC with post-column permanganate chemiluminescence detection for screening of antioxidants in green and black tea samples, but it was not directly compared with on-line DPPH• and ABTS•+ protocols [29]. Furthermore, using six model phenolic compounds, we have tentatively shown that the chemiluminescence intensity upon reaction with acidic potassium permanganate could be used to predict their ability to attenuate oxidative stress in cultured human myoblasts based upon *in vitro* cell culture assays [23]. However, it should be borne in mind that, as for all *in vitro* antioxidant assays, further biological testing of candidate compounds would be required to confirm the activity predicted by this post-column reaction.

In a subsequent investigation, permanganate chemiluminescence detection and the DPPH• radical decolouration assay were simultaneously employed for the evaluation and comparison of antioxidants in espresso coffee samples, by dividing the post-column HPLC eluate (50:50 ratio, controlled with a pressure regulator) [24]. The results from the two approaches were similar, but some differences in selectivity were observed. Chromatograms generated with the chemiluminescence detection contained more peaks, which was attributed to the greater sensitivity of the reagent towards minor, readily oxidisable sample components [24].

Although these studies provide insight into the potential of acidic potassium permanganate chemiluminescence as an antiox-

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