Talanta 130 (2014) 221–225

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

Ethanol as an alternative to formaldehyde for the enhancement of manganese(IV) chemiluminescence detection

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ARTICLE INFO

Article history:
Received 19 May 2014
Received in revised form
30 June 2014
Accepted 1 July 2014
Available online 9 July 2014

keywords:
Manganese(IV)
Chemiluminescence
Flow injection analysis
High performance liquid chromatography
Thiols
Disulfides

ABSTRACT

Previous applications of manganese(IV) as a chemiluminescence reagent have required the use of formaldehyde to enhance the emission intensity to analytically useful levels. However, this known human carcinogen (by inhalation) is not ideal for routine application. A wide range of alternative enhancers have been examined but to date none have been found to provide the dramatic increase in chemiluminescence intensities obtained using formaldehyde. Herein, we demonstrate that ethanol offers a simple, safe and inexpensive alternative to the use of formaldehyde for manganese(IV) chemiluminescence detection, without compromising signal intensity or sensitivity. For example, chemiluminescence signals for opiate alkaloids using 50–100% ethanol were 0.8–1.6-fold those using 2 M formaldehyde. This innocuous alternative enhancer is shown to be a particularly effective for the direct detection of thiols and disulfides by manganese(IV) chemiluminescence, which we have applied to a simple HPLC procedure to determine a series of biomarkers of oxidative stress.

1. Introduction

The 'soluble' (colloidal) manganese(IV) reagent prepared by adding freshly precipitated manganese dioxide to 3 M orthophosphoric acid [1,2] has been employed for chemiluminescence detection of a variety of inorganic and organic compounds [3–5]. The emitting species in these reactions has been identified as manganese(II) [4,6]. This emitter is also formed in reactions with acidic potassium permanganate [7,8], but these two chemiluminescence oxidants exhibit considerably different selectivity [4,5,8], where manganese(IV) produces light with a much wider range of analytes. Recent notable applications of this chemiluminescence reagent include a simple HPLC procedure to determine the ratio of glutathione to its disulfide in biological fluids to assess oxidative stress (without the need for pre-column analyte derivatization or disulfide bond reduction) [9], and a rapid flow injection analysis (FIA) approach to establish total phenolic antioxidants in plant derived samples [10,11].

The analytical application of manganese(IV) chemiluminescence has to date required the use of formaldehyde (between 0.2 M and 3.0 M), which increases the chemiluminescence intensity by orders of magnitude [3,4]. However, formaldehyde is a carcinogen (by inhalation) and not ideal for routine use. Aside from the obvious concerns for the user's safety, such classification also leads to practical issues involving ordering, storage and disposal. Consequently, a variety of other compounds have been examined as potential enhancers (including other aldehydes, formic acid, sodium polyphosphates, surfactants, β-cyclodextrin, sodium thiosulfate, sodium sulfite and quinine [12–14]), but these species have generally given only poor enhancement of the light-producing reactions with manganese(IV).

Considering that, in aqueous solution at room temperature, formaldehyde is largely hydrated to form methanediol [15], we have explored a series of simple alcohols and related compounds as possible enhancers of manganese(IV) chemiluminescence. This has revealed that the addition of ethanol can provide superior emission intensities to that of the conventional formaldehyde enhancer for manganese(IV) chemiluminescence detection.

2. Materials and methods

2.1. Flow injection analysis (FIA)

The manifold was constructed from a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, NSW, Australia) with bridged PVC or silicone pump tubing (white/white, 1.02 mm i.d., DKSH,

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http://dx.doi.org/10.1016/j.talanta.2014.07.002
0039-9140/

Queensland, Australia), PTFE manifold tubing (0.8 mm i.d., Cole-Parmer Instrument Company, Illinois, USA) and a six-port injection valve (Vici 04W-0192L Valco Instruments, Texas, USA) equipped with a 70 μL sample loop. A custom built flow-cell (a tight coil of 0.8 mm i.d. PTFE tubing) was mounted flush against an extended range photomultiplier tube (Electron Tubes model 9828SB, ETP, NSW, Australia) and encased in a light-tight housing. The output signal was recorded using an e-corder 410 data acquisition system (eDAQ, NSW, Australia). To facilitate the incorporation of enhancers, analytes were injected into a carrier stream (deionized water) which merged with a stream of either deionized water or enhancer solution at a confluence point located at the entrance of a short (15 cm) reaction coil. This stream was then combined with the reagent just prior to entry into the flow cell. The flow-rate was 3.5 mL min^{-1} per line.

2.2. High performance liquid chromatography (HPLC)

An Agilent Technologies 1260 series liquid chromatography system was used, with quaternary pump, solvent degasser system, and autosampler (Agilent Technologies, Forest Hill, Victoria, Australia), and an Alltech Alltima C18 column (250 mm × 4.6 mm i.d., 5 μm) with Alltima C18 guard column, at room temperature, with an injection volume of 100 μL and a flow rate of 1 mL min^{-1}. Isocratic elution was performed with 97% solvent A (deionized water adjusted to pH 2.57 with trifluoroacetic acid) and 3% solvent B (methanol). An analog to digital interface box (Agilent Technologies) was used to convert the signal from the chemiluminescence detector. Before use in the HPLC system, all sample solutions and solvents were filtered through a 0.45 μm nylon membrane. For post-column chemiluminescence measurements the column eluate (1 mL min^{-1}) and enhancer (1 mL min^{-1}) were merged at a T-piece located 10 cm from the entrance of the detector. This stream was then combined with the reagent (1 mL min^{-1}) upon entering the flow-cell. A GloCel chemiluminescence detector (Global FIA, WA, USA) containing a dual-inlet serpentine flow-cell [16] and photomultiplier tube (model 9828SB, ETP, NSW, Australia) set at a constant voltage of 900 V from a stable power supply (PM20D, ETP) was utilized in this system. The reagent and enhancer solutions were propelled by a 12 × 6 Dual Piston Pump (PEEK) (Scientific Systems, PA, USA) equipped with a pulse damper and self-flushing pump heads.

2.3. UV–visible spectrometry

The absorption of reagent solutions was measured using a Cary 300 Bio UV–vis Spectrophotometer (Varian Australia, Mulgrave, Victoria, Australia) with a 1 cm quartz cuvette, a scan rate of 600 nm min^{-1} and a bandwidth of 1 nm. Spectra were recorded over the range 200–750 nm. Equal volumes of reagent, enhancer and deionized water were combined in the cuvette immediately prior to recording the first spectrum. Spectra were then recorded every 3 min for 120 min. The mixture remained untouched until the final spectrum was recorded.

2.4. Chemicals

Deionized water and analytical grade reagents were used unless otherwise stated. Chemicals were obtained from the following sources: *N*-acetylcysteine (Nacys), chlorotyrosine, clozapine, cysteine (Cys), cystine (Cyss), dopamine, 1,2-ethanediol, flavone, fluphenazine, glutathione (GSH), glutathione disulfide (GSSG), glyoxal, hesperetin, homocysteine (Hcys), homocystine (Hcyss), kynurenine, methionine, prochlorperazine, rosmarinic acid, sodium formate, sodium polyphosphate (+200 mesh), sodium thiosulfate, trifluoroacetic acid, tryptophan, *m*-tyrosine, *o*-tyrosine and *p*-tyrosine from Sigma-Aldrich (New South Wales, Australia); ethanol, formaldehyde (37%), orthophosphoric acid (85%), potassium permanganate, 1-propanol and 2-propanol from Chem-Supply (South Australia, Australia); codeine, heroin, morphine, noscapine, oripavine and thebaine from GlaxoSmtihKline (Victoria, Australia); methanol and sulfuric acid from Merck (Victoria, Australia); soluble starch from Ajax Finechem (New South Wales, Australia) and formic acid from Hopkin and Williams (Essex, England). With some exceptions, analyte stocks were prepared in deionized water at 1×10^{-3} M. Sulfuric acid (~10 drops in 250 mL) was added to the solutions of morphine, codeine, thebaine, oripavine, noscapine, chlorotyrosine, *m*-tyrosine, *o*-tyrosine, *p*-tyrosine, clozapine, fluphenazine and prochlorperazine to aid dissolution. The thiol and disulfide compounds were prepared in either deionized water adjusted to pH 2.8 with formic acid (for FIA) or in mobile phase (for HPLC). Flavone, hesperetin and rosmarinic acid were prepared in methanol. Heroin was prepared in 0.1% acetic acid. Stock solutions were diluted daily in deionized water to working concentrations.

2.5. Manganese(IV) reagent

The reagent was prepared as previously described [3], based on the method of Jáky and co-workers [1,2]. Freshly precipitated manganese dioxide, obtained by reduction of potassium permanganate with excess sodium formate, was collected by vacuum filtration and washed with deionized water. Subsequently, 0.6 g of the wet material was added to 500 mL of orthophosphoric acid (3 M) and sonicated for 30 min. The colloid was heated at 80 °C for 1 h, cooled to room temperature, and the concentration determined by iodometric titration. The stock manganese(IV) reagent was diluted daily to 5×10^{-4} M using 3 M orthophosphoric acid.

2.6. Acidic potassium permanganate reagent

Potassium permanganate (1×10^{-3} M) was dissolved in a 1% (m/v) solution of sodium polyphosphate. The pH was then adjusted to 2.5 by the dropwise addition of sulfuric acid.

3. Results and discussion

3.1. Preliminary investigations

Using FIA methodology, the enhancement of manganese(IV) chemiluminescence by formaldehyde (2 M) was compared with that of three simple alcohols: methanol, ethanol and 2-propanol, as well as acetonitrile (100%), against the response with no enhancer (DI water). Four opiate alkaloids (morphine, codeine, thebaine and oripavine), which have previously been determined with this chemiluminescence reagent [3], were used as model analytes. In agreement with previous reports of quenching of the chemiluminescence responses with manganese-based reagents by HPLC column eluates containing acetonitrile [9,17], no significant response from the analytes was observed when this solvent was added (Fig. 1). Methanol increased the chemiluminescence intensities to a small extent (115–173 fold), isopropanol provided intensities comparable to those obtained with formaldehyde (2512–3733 and 2537–3518 fold, respectively), whereas ethanol afforded superior levels of enhancement for all four analytes (3755–5765 fold). Using ethanol as an enhancer therefore gave signals that were 48–64% greater than using 2 M formaldehyde.

Several other potential enhancers that were structurally related to formaldehyde or methanediol, including 1,2-ethanediol, glyoxal and 1-propanol, were compared in a similar manner (see Table S1 in Supporting information), but none provided increases in analyte responses that were comparable with those obtained

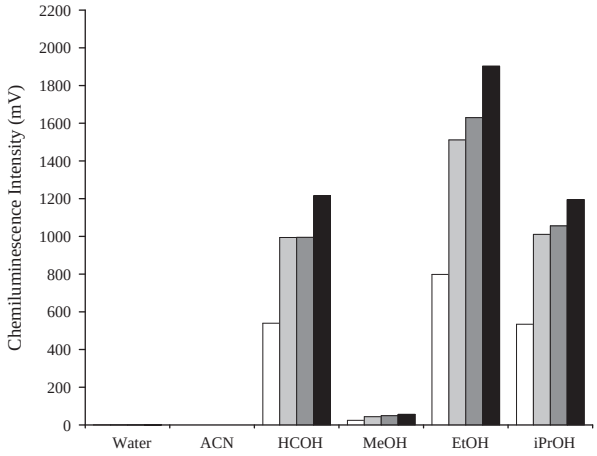

Fig. 1. Chemiluminescence intensities for four opiate alkaloids (5×10^{-6} M) with colloidal manganese(IV) and potential enhancers (acetonitrile (ACN), formaldehyde (HCOH), methanol (MeOH), ethanol (EtOH) and isopropanol (iPrOH)), using FIA methodology. White bars: morphine; light gray: oripavine; dark gray: codeine; black: thebaine.

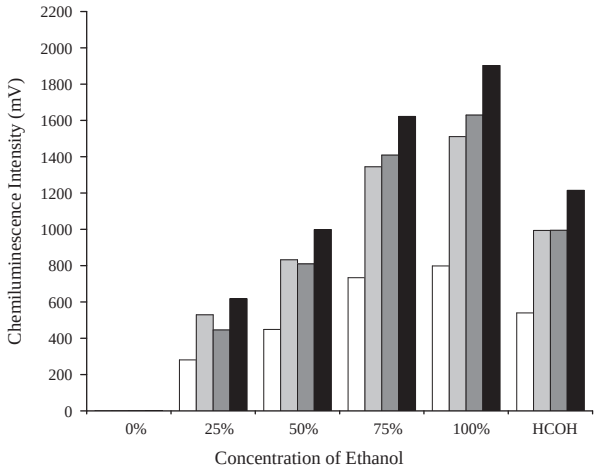

Fig. 2. Chemiluminescence intensities for four opiate alkaloids (5×10^{-6} M) with colloidal manganese(IV) using different concentrations of ethanol (or 2 M formaldehyde, HCOH) to enhance the emission intensity, using FIA methodology. White bars: morphine; light gray: oripavine; dark gray: codeine; black: thebaine.

using ethanol. Interestingly, glyoxal provided a moderate signal enhancement, but the background noise produced by the continuous reaction between enhancer and reagent was exceedingly high, making it impractical for use. Isomers 1-propanol and 2-propanol gave similar levels of enhancement, but 1-propanol exhibited 8-fold greater background noise (Table S2). Although the most effective concentration of formaldehyde has generally been reported to be between 0.2 M and 3.0 M, the enhancement by ethanol increased up to a 100% concentration in the enhancer stream (Fig. 2). Ethanol concentrations in the range of 50–100% gave analyte responses comparable (0.8–1.6-fold) to the commonly used 2 M formaldehyde enhancer.

To determine if the relative enhancement observed using ethanol and formaldehyde was dependent on the structure of the target analyte, the responses of 20 compounds were compared using FIA methodology (Fig. 3). The tested compounds included a range of antioxidants, amino acids, neurotransmitters and pharmaceuticals. The corresponding response with the related permanganate (manganese(VII)) chemiluminescence reagent [8,18] was also examined. This comparison showed: (i) the much broader selectivity of the manganese(IV) reagent (with either enhancer) compared to that of the permanganate reagent, despite the fact that the light generated has been shown to emanate from a common manganese(II) emitter [6,7]; (ii) ethanol generally provided superior enhancement of the chemiluminescent reactions with manganese(IV) compared to formaldehyde, with the exception of the bioflavonoid hesperetin; (iii) the use of ethanol instead of formaldehyde results in some changes in reagent selectivity. The difference in response was 20–60% for most compounds, but it was significantly greater for glutathione (544%), glutathione disulfide (212%), and the thioether methionine (200%). The improved selectivity towards thiol and disulfide compounds with the ethanol enhancer is particularly attractive for their determination in biological samples as a measure of oxidative stress [9].

3.2. Examination of the background reaction

Although the mechanism of enhancement of manganese-based chemiluminescence reagent systems by formaldehyde is yet to be fully elucidated [4,8,18], in the case of permanganate it is thought to in part involve the *in situ* generation of higher concentrations of the manganese(III) precursor to the emitter [19]. For both the permanganate and the manganese(IV) reagents, the complete reduction by formaldehyde to form the manganese(II) emitter can be observed as a background emission from the reagent and enhancer in the absence of any analyte (Table S2). The UV–visible absorption spectra of these reaction mixtures showed slow decreases in the broad characteristic band of manganese(IV) (Fig. 4a, and Fig. S1 in Supplementary Information). At wavelengths below ~250 nm, variations were seen for different enhancers because of their absorption within this region (see blue traces in Fig. S1). Throughout the reactions, the colloid remained stable (no flocculation occurred).

The differences in the reaction kinetics can be more easily discerned by examining the change in absorption over time at 420 nm (Fig. 4b). The rates of reaction of manganese(IV) with formaldehyde and ethanol are significantly greater than those with either methanol or 2-propanol. Moreover, the spectral distribution of the chemiluminescence from the reaction of manganese(IV) and codeine using ethanol as the enhancer is identical to that using formaldehyde (Fig. S2), both of which match the characteristic luminescence of manganese(II) [7]. It can therefore be concluded that, similar to the chemiluminescent reactions with permanganate [19], the superior enhancing effects of formaldehyde and ethanol compared to methanol and propanol with the manganese(IV) reagent are (in part) due to the more rapid generation of manganese(III) and manganese(II) (known to catalyze reactions with manganese oxidants [1,20,21]). However, this does not explain the considerably greater enhancing effect of 2-propanol than methanol, nor does it account for the absence of significant enhancement from other reducing agents [12–14]. It is thus likely that an additional mode of enhancement is involved, such as the protection of the excited state emitting species from non-radiative deactivation, similar to one action of sodium polyphosphate in the chemiluminescent reduction of permanganate [22].

3.3. HPLC separation and figures of merit

To demonstrate the viability of ethanol as an alternative enhancer of manganese(IV) chemiluminescence detection for the HPLC determination of biologically important thiols and disulfides, we compared the two enhancers using the separation conditions described by McDermott and co-workers [9]. The procedure involves the use of a simple isocratic mobile phase consisting of 97% deionized water adjusted to pH 2.57 with trifluoroacetic acid and 3% methanol, which enables baseline resolution of each compound in under 20 min. The enhancer was added by merging

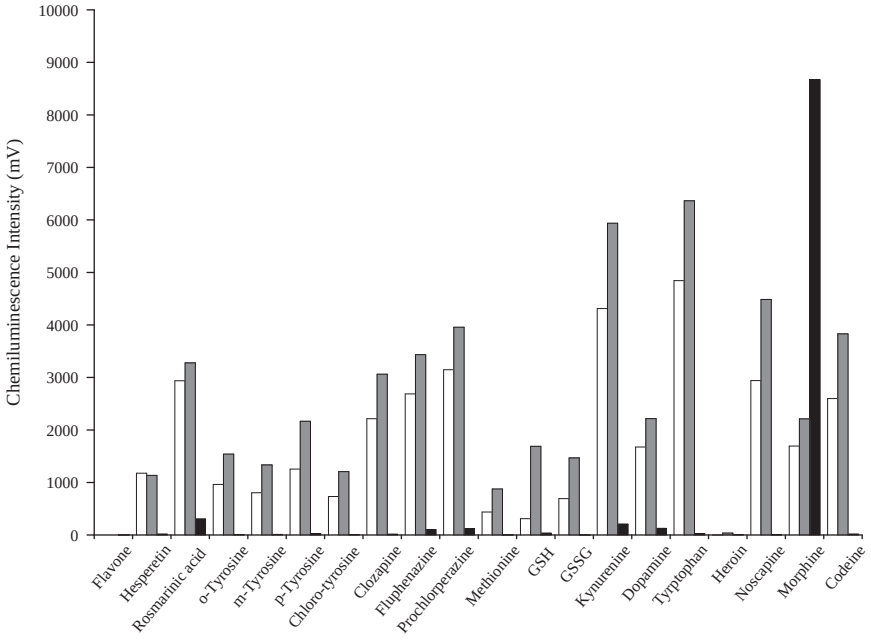

Fig. 3. Chemiluminescence intensities for 20 compounds (5×10^{-6} M) with the manganese(IV) reagent and formaldehyde enhancer (white bars); the manganese(IV) reagent and ethanol enhancer (gray bars); and a permanganate reagent (black bars), using FIA methodology.

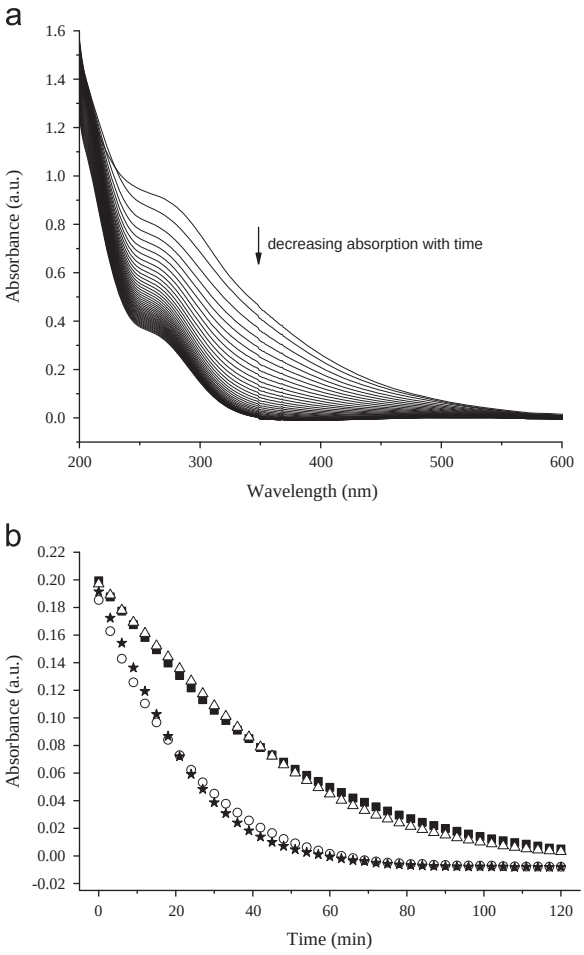

Fig. 4. Absorbance at 420 nm over time plots for: formaldehyde (black stars), methanol (black squares), ethanol (white circles) and 2-propanol (white triangles).

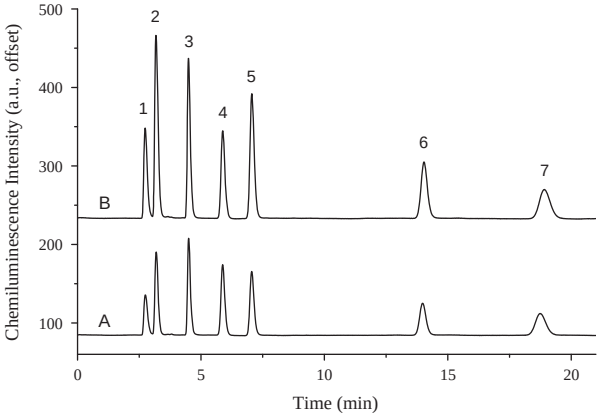

Fig. 5. HPLC separation and chemiluminescence detection of a mixture of seven biologically important thiols and disulfides. 1: cystine, 2: cysteine, 3: homocysteine, 4: homocystine, 5: glutathione, 6: *N*-acetylcysteine, and 7: glutathione disulfide (1×10^{-5} M, 100 μL injection volume), using the manganese(IV) reagent with (A) 2 M formaldehyde or (B) ethanol as an enhancer.

a flowing stream with the column-eluate at a confluence point 10 cm from the flow-cell entrance. The manganese(IV) reagent then merged with this mixture within a dual-inlet serpentine flow-cell [16].

The ethanol enhancer provided peak areas between 27% and 127% greater than the formaldehyde enhancer (Fig. 5), which resulted in a slight change in the relative peak heights of the seven analytes. Calibration curves were prepared using 13 standard solutions between 1×10^{-8} M and 1×10^{-5} M (Table 1). In each case, an approximately linear relationship between peak area and analyte concentration was obtained, with limits of detection between 5×10^{-8} M and 1×10^{-7} M. These figures are similar to those previously published for manganese(IV) chemiluminescence using a formaldehyde enhancer [9]. Although the absolute chemiluminescence responses (peak areas) for the analytes were larger

Table 1
Analytical figures of merit.

	Retention					
Analyte	Time (min)	% RSD	R^2	Linear range (μM)	% RSD	LOD (M)
Cyss	2.73	0.11	0.996	0.05–10	2.99	5×10^{-8}
Cys	3.17	0.08	0.999	0.05–10	4.64	5×10^{-8}
Hcys	4.41	0.03	0.999	0.05–10	4.03	5×10^{-8}
Hcyss	5.72	0.20	0.998	0.05–10	3.24	5×10^{-8}
GSH	6.88	0.12	0.999	0.05–10	4.91	7×10^{-8}
Nacys	13.62	0.20	0.997	0.05–10	4.89	7×10^{-8}
GSSG	17.75	0.51	0.999	0.05–10	2.34	1×10^{-7}

with ethanol than formaldehyde, the alcohol produced a higher continuous background signal (Table S2), the slightly poorer stability of which resulting in similar limits of detection.

4. Conclusions

Ethanol afforded greater levels of enhancement than formaldehyde for the majority of analytes with the manganese(IV) chemiluminescence reagent. Although, a stream containing 100% ethanol provided the greatest enhancement, concentrations between 50% and 100% were comparable to the previously used formaldehyde enhancer. The ethanol enhancer was particularly suited to the detection of compounds containing a thiol or disulfide group, with limits of detection similar to those previously reported using a formaldehyde enhancer. Consequently, ethanol offers a simple, safe and inexpensive alternative to the use of formaldehyde, without compromising signal intensity or detection sensitivity. Given that the manganese(IV) reagent could not previously provide sufficiently sensitive detection without the use of a carcinogenic enhancer, the replacement of this compound with ethanol will enable wider application of this useful chemiluminescence detection system.

Acknowledgments

The authors thank Deakin University and the Australian Research Council (DP14100439) for funding.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.07.002.

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