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Short communication

Chemiluminescence detection of amino acids and related compounds using acidic potassium permanganate, manganese(IV) or tris(2,2′-bipyridine)ruthenium(III)

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ABSTRACT

We have explored the chemiluminescence response of amino acids and related compounds (including the tripeptide glutathione, and disulfides: cystine, homocystine and glutathione disulfide) with several new adaptations of the permanganate and tris(2,2′-bipyridine)ruthenium(III) ($[Ru(bipy)_3]^{3+}$) reagents and a recently developed colloidal manganese(IV) system. The selectivity of the permanganate reagent can be directed towards tyrosine or thiol compounds like cysteine, homocysteine and glutathione by manipulating reaction conditions (providing limits of detection of 4 nM tyrosine and 5 nM glutathione). Colloidal Mn(IV) produced measureable responses with all analytes, but was most suitable for tryptophan, tyrosine, thiols and disulfides, including α-lipoic acid and dihydrolipoic acid, for which the limits of detection were 30 nM and 20 nM, respectively. A stabilised form of $[Ru(bipy)_3]^{3+}$, prepared by oxidising $[Ru(bipy)_3](ClO_4)_2$ in acetonitrile, exhibited similar selectivity to that of the conventional aqueous reagent. The $[Ru(bipy)_3]^{3+}$ reagent was effective for the detection of secondary amino acids such as proline and hydroxyproline, as well as the disulfide, homocystine.

1. Introduction

An extensive range of analytical methodology for amino acids has been developed [1–4], but the on-going exploration of new, alternative modes of detection is driven by the desire for faster, simpler and/or more sensitive analysis in many fields [5–7]. In studies of the amino acids and short peptides involved in cellular oxidative defences, for example, the replacement of traditional derivatisation-based spectroscopic detection in HPLC assays, with 'direct' post-column modes of detection (such as mass spectrometry [8] and chemiluminescence [9]) has shortened analysis times, but more importantly, it has reduced the deleterious artificial effects of sample pre-treatment on the cellular redox state.

A previous paper from our group described a series of chemiluminescence reagents that exhibited considerable selectivity towards specific amino acids [10], which we exploited in simple flow analysis procedures for the determination of Arg, His, Phe, Pro, Trp and Tyr [10], and subsequently extended to the detection of peptides such as angiotensins [11] and glutathione (GSH) [12] after chromatographic separations. In recent years, there have been several major advances in two of the reagent systems (acidic potassium permanganate and tris(2,2′-bipyridine)ruthenium(III) ($[Ru(bipy)_3]^{3+}$)), in terms of their stability [13,14] and sensitivity [14,15], and the understanding of their light producing pathways [16,17]. Moreover, we have developed a new chemiluminescence reagent based on a manganese(IV) colloid [18], which produces light with a wide range of compounds including several amino acids [9,19,20]. In light of these advances and the mounting interest in the detection of underivatised analytes in applications such as amino acid analysis and assessment of cellular oxidative stress, in this paper we compare the relative response from 26 amino acids and related compounds (including the tripeptide GSH, and disulfides: cystine (CYSS), homocystine (HCYSS) and GSSG) with conventional and new adaptations of these chemiluminescence reagents. This includes the first examination of the stabilised $[Ru(bipy)_3]^{3+}$ reagent, enhanced permanganate reagents and the colloidal manganese(IV) reagent for the detection of a wide range of amino acids and related species.

2. Experimental

2.1. Flow injection analysis (FIA)

The instruments were constructed from a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, New South Wales,

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Australia) with bridged PVC pump tubing (1.02 mm i.d., DKSH, Queensland, Australia) and a 6-port injection valve (Vici 04W-0192L; Valco Instruments, Texas, USA) equipped with a 70 μL sample loop. A GloCel detector (Global FIA, Washington, USA) with a dual-inlet serpentine flow-cell and extended range photomultiplier module (Electron Tubes model P30A-05; ETP, New South Wales, Australia) [21] was used for the permanganate and manganese(IV) experiments, and an in-house fabricated detector containing an aluminium-backed coiled-tubing flow-cell and extended-range photomultiplier module within a light-tight housing [22] was used for the $[Ru(bipy)_3]^{3+}$ experiments. All tubing entering and exiting the detectors was black PTFE (0.76 mm i.d.; Global FIA). The output signal from the photomultiplier module was recorded with an e-corder 410 data acquisition system (eDAQ, New South Wales, Australia). Analytes were injected into a water carrier stream that merged with the reagent, unless otherwise stated.

2.2. Chemicals and reagents

Deionised water and analytical grade reagents were used unless otherwise stated. Chemicals were obtained from the following sources: formaldehyde (37%), orthophosphoric acid (85%), and potassium permanganate from Chem-Supply (South Australia, Australia); lead dioxide, potassium iodide and sodium perchlorate from Ajax Finechem (New South Wales, Australia); sulfuric acid from Merck (Victoria, Australia); formic acid from Hopkin and Williams (Essex, England); perchloric acid (70% m/v) from Univar (New South Wales, Australia); aqueous soluble starch from Fisons Scientific Equipment (Loughborough, England); acetonitrile from Burdick & Jackson (Michigan, USA); and tris(2,2′-bipyridine)ruthenium(II) dichloride hexahydrate from Strem Chemicals (Minnesota, USA). All other chemicals were purchased from Sigma-Aldrich (New South Wales, Australia), except for dihydrolipoic acid and a series of *N*-phenylamides, which were prepared from α-lipoic acid, as previously described [23]. Analyte solutions were prepared daily.

The 'standard' permanganate reagent (**Reagent A**) [15] was prepared by dissolving potassium permanganate (1 mM) in a 1% m/v sodium polyphosphate solution and adjusting to pH 2.5 with sulfuric acid.

The permanganate reagent utilised in our previous study for the selective detection of tyrosine (**Reagent B**) [10] was prepared by dissolving potassium permanganate (3.1 mM) in a 1% m/v sodium polyphosphate solution and adjusting to pH 6.75.

The 'enhanced' permanganate reagent (**Reagent C**) [15] was prepared by dissolving potassium permanganate (1.9 mM) in a 1% m/v sodium polyphosphate solution, adjusting to pH 2.5, and then adding sodium thiosulfate (0.6 mM), using a small volume of a 100 mM stock solution.

The permanganate reagent optimised for the detection of thiols (**Regent D**) [12] was prepared by dissolving potassium permanganate (0.25 mM) in a 1% m/v sodium polyphosphate solution and adjusting to pH 3. Analytes (0.1 μM) were injected into a carrier stream (deionised water adjusted to pH 2.8 with formic acid) that merged with the reagent.

Soluble manganese(IV) (**Reagent E**) was prepared as previously described [9,20]. The stock manganese(IV) solution was diluted daily to 0.5 mM using orthophosphoric acid (3 M). Formaldehyde was filtered and diluted to 2 M with deionised water. Analytes were injected into a deionised water carrier stream that was combined with a 2 M formaldehyde line, before merging with the reagent.

The $[Ru(bipy)_3]^{3+}$ reagent (**Reagent F**) [10] was prepared by oxidising 0.05 mM tris(2,2'-bipyridine)ruthenium(II) with 0.2 g/100 mL lead dioxide in 0.02 M sulfuric acid. Following the change in colour from orange to emerald green (~5 min with stirring), the excess oxidant was filtered from the solution using a 0.45 μm syringe-tip filter. Using a three-line FIA manifold, a continuously flowing analyte stream (5 μM) was merged with a sodium tetraborate buffer (100 mM, pH 10) and then combined with a deionised water carrier immediately prior to entering the coiled-tubing flow-cell. The reagent (0.05 mM) was injected into the carrier stream.

The 'stabilised' $[Ru(bipy)_3]^{3+}$ reagent (**Reagent G**) [13] was prepared as follows: $[Ru(bipy)_3]Cl_2$ was treated with sodium perchlorate in aqueous solution to yield a $[Ru(bipy)_3](ClO_4)_2$ precipitate, which was collected by vacuum filtration, washed twice with ice water, and dried over phosphorus pentoxide for 24 h. The reagent solution was prepared by dissolving the crystals in acetonitrile containing 0.05 M perchloric acid. Lead dioxide (0.2 g/100 mL) was added to form the blue–green $[Ru(bipy)_3]^{3+}$. Initial tests with this reagent were performed using the conditions described for **Reagent F**. In subsequent experiments, optimised for the detection of thiols and disulfides, the analyte (10 μM) was injected into a carrier stream (deionised water adjusted to pH 2.8 with formic acid), which merged with a phosphate buffer (40 mM, pH 8) stream and then the reagent (1 mM).

3. Results and discussion

3.1. Acidic potassium permanganate

An early investigation of the chemiluminescent oxidation of twelve amino acids (Ala, Asn, Asp, Cys, Glu, His, Lys, Met, Phe, Ser, Thr and Trp) with permanganate [24] showed that although light was detected in each case, the intensities ranged over three orders of magnitude, with the greatest responses produced by Glu and Asp. Subsequent studies employing acidic conditions included the detection of Tyr [25,26] and Trp [27,28], but without comparison to other amino acids. During this time, the characteristic red emission from these reactions was ascribed to the Mn(II) product of the reaction [25], which has only recently been confirmed by direct spectroscopic evidence [29]. In 2003, our research group [10] optimised an acidic permanganate reagent containing sodium polyphosphate (a commonly used enhancer [30]) for the detection of Trp and Tyr after a low-pressure chromatographic separation. No significant emissions were detected from the other 18 analytes investigated [10].

In the current study, we have used FIA to examine the response of 26 analytes with a 1 mM permanganate reagent containing 1% m/v sodium polyphosphate and adjusted to pH 2.5 with sulfuric acid (**Reagent A**; Fig. 1(i)), as a compromise between the conditions providing the greatest intensities for Trp ($pH \leq 2$) and thiol compounds (pH 3; Fig. 2). With this reagent, Trp and Tyr produced the largest signals, followed by the thiols, and then Lys. Much smaller signals were recorded for all other analytes. We previously found that an increase in reagent pH (to between 4 and 8) eliminated the signal from Trp, whilst increasing the response for Tyr [10]. Examination of the 24 other compounds under the conditions optimised for the detection of Tyr (**Reagent B**) identified only Pro and Hyp as minor positive interferences (data not shown).

A preliminary partial reduction of permanganate reagents (creating high concentrations of the Mn(III) precursor to the emitter) can enhance the chemiluminescence from phenolic compounds [14,15], which offers an alternative approach to derive greater selectivity for Tyr over other amino acids. In our current study, the response for Tyr using an 'enhanced' reagent (**C**) was found to be at least 20-fold greater than any other analyte tested under the same conditions (Fig. 1(ii)), and 70-fold greater than the response for Tyr using Reagent A (Fig. 1(i)) or B. We attribute the superior selectivity towards tyrosine under these

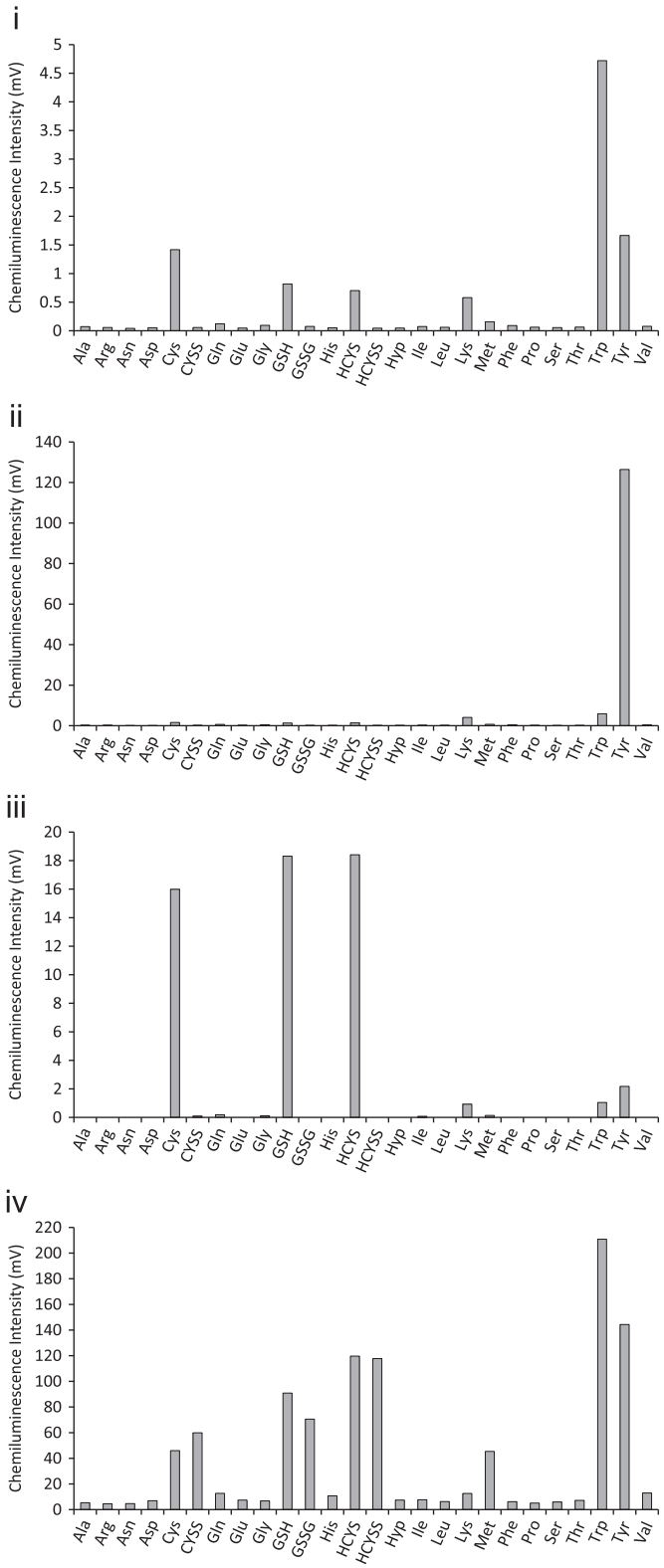

Fig. 1. Relative chemiluminescence intensities with permanganate: (i) Reagent A, (ii) Reagent C, and (iii) Reagent D; and colloidal manganese(IV): (iv) Reagent E, using FIA (with an analyte concentration of 10 μM). The average RSD for replicate injections of analytes that gave significant signals was 2.1%, 1.2%, 0.7% and 1.9%, using Reagents A, C, D and E.

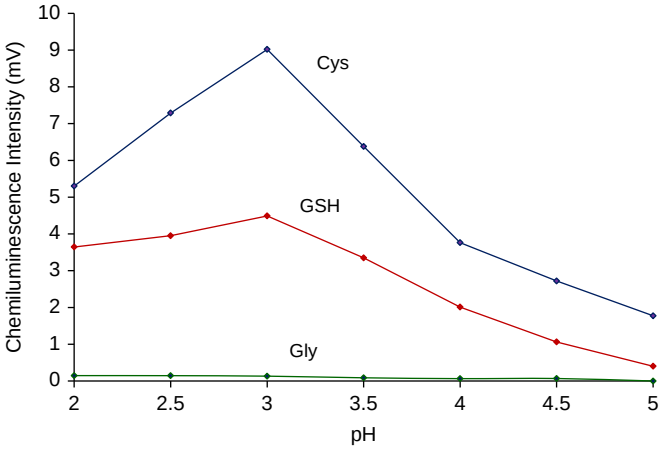

Fig. 2. Relative chemiluminescence intensity of Cys, GSH and Gly (50 μM) with acidic potassium permanganate (1 mM), using flow injection analysis methodology. The reagents were prepared by dissolving $KMnO_4$ in a 1% m/v sodium polyphosphate solution and adjusting the pH with sulfuric acid.

conditions to differences in the reactivity of phenolic and non-phenolic species with permanganate and Mn(III) [31], where a higher Mn(III) concentration in the reagent results in a much greater increase in the rate of reaction (and therefore the maximum chemiluminescence intensity) with phenolic analytes, particularly those that otherwise react slowly with permanganate [15]. Using Reagent C, the limit of detection of 4 nM was better than all previously reported for this analyte using permanganate chemiluminescence.

Fig. 1(i) also shows the potential of the acidic potassium permanganate reagent for the detection of thiols. Li and co-workers reported the determination of thiols such as Cys, GSH, *N*-acetylcysteine and captopril based on the chemiluminescence reaction with permanganate and quinine [32]. This system actually contains two light-producing pathways: the reduction of permanganate to Mn(II)*, and the oxidation of the thiols to form an excited intermediate capable of transferring energy to the quinine fluorophore [33]. Under the conditions employed by Li et al. [32], the emission from quinine dominates, but the pathway to Mn(II)* can be promoted by the addition of sodium polyphosphate (rather than quinine) [33], which stabilises the Mn(III) intermediate and forms protective cage-like structures around the emitter [16].

Unlike the reagent developed for the detection of Tyr, the optimal conditions for thiols were at pH 3 and a permanganate concentration of 0.25 mM (**Reagent D**). This approach is not only simpler (because the enhancer can be added to the reagent solution rather than merging with the analyte), but also provides greater sensitivity. In a related study [12], we have utilised this detection system in a HPLC procedure for GSH in cultured muscle cells (the limit of detection for GSH was 0.5 μM), which was also used to quantify GSSG by incorporating thiol blocking and disulfide bond reduction steps. In the current investigation, we used FIA methodology to compare the response from the 26 amino acids and related species (Fig. 1(iii)). Each analyte was injected into a carrier stream (deionised water adjusted to pH 2.8 with formic acid) that merged with the reagent. Compared to **Reagent A**, the responses of the three thiols (Cys, GSH, HCYS) were increased by at least an order of magnitude, whilst the response for most other analytes decreased. Under these conditions, we obtained a limit of detection for GSH of 5 nM, compared to 65 nM reported by Li et al. [32].

3.2. Manganese(IV)

Like permanganate, colloidal Mn(IV) can produce an electronically excited Mn(II)* species upon reaction with various oxidisable compounds [18,20,34]. Chemiluminescence from reactions

between Mn(IV) and several amino acids has been noted [9,19,20] and we previously used this reagent in a HPLC procedure for the determination of GSH and GSSG in whole blood samples [9], but until now, the relative responses of different amino acids was not known. To establish this relationship, we used FIA methodology, where the analytes were injected into a water carrier that was combined with 2 M formaldehyde, before merging with the Mn(IV) reagent (**E**). Compared to the permanganate reagents, Mn(IV) exhibited broader selectivity. A measureable response was obtained from all analytes (Fig. 1(iv)), with those detected by Reagents C (Cys, GSH, HCYS) and D (Trp, Tyr) as well as disulfide and thioether compounds (CYSS, GSSG, HCYSS, Met) producing the largest signals. The broader applicability of the Mn(IV) reagent may in part arise from the heterogeneous reaction conditions, where absorption of the analytes onto the surface of Mn(IV) nanoparticles promotes electron transfer to the metal centre [35]. Although colloidal Mn(IV) has been identified as an autocatalytic intermediate or product of reactions between permanganate and various organic compounds, including amino acids [36], the accumulation of Mn(IV) in reactions with Reagents **A**–**D** is precluded by the large excess of sodium polyphosphate in the solutions [16].

To further explore the potential of Mn(IV) and permanganate for the detection of thiols and disulfides, we examined the response from the reduced and oxidised forms of α-lipoic acid (a therapeutic agent that affects cellular metabolic processes and redox status [37]), and five *N*-phenylamide derivatives (Fig. 3) that were recently examined for their ability to block androgen-stimulating proliferation of human prostate cancer cells [23]. With Mn(IV), large signals were obtained from all analytes (Fig. S1(i)). With permanganate (Reagent **D**), α-lipoic acid and its *N*-phenylamide derivatives produced signals that were 2- to 5-fold greater than CYSS, but considerably lower than the thiol compounds (Fig. S1(ii)). In both cases, the greatest signal was produced by dihydrolipoic acid, which contains two thiol groups. Limits of detection for α-lipoic acid and dihydrolipoic acid using FIA were 0.03 μM and 0.02 μM with Mn(IV), and 0.3 μM and 0.08 μM with permanganate. The relative standard deviations of replicate injections at analyte concentrations of 1.0 μM with the Mn(IV) reagent and 2.5 μM with permanganate were between 0.6% and 2.3%. During the preparation of this manuscript, Wolyniek et al. [38] reported the determination of α-lipoic acid based on the chemiluminescence reaction with potassium permanganate in an acidic polyphosphate solution. The emission intensity was significantly enhanced by formaldehyde, which enabled a limit of detection for α-lipoic acid of 0.02 μM [38].

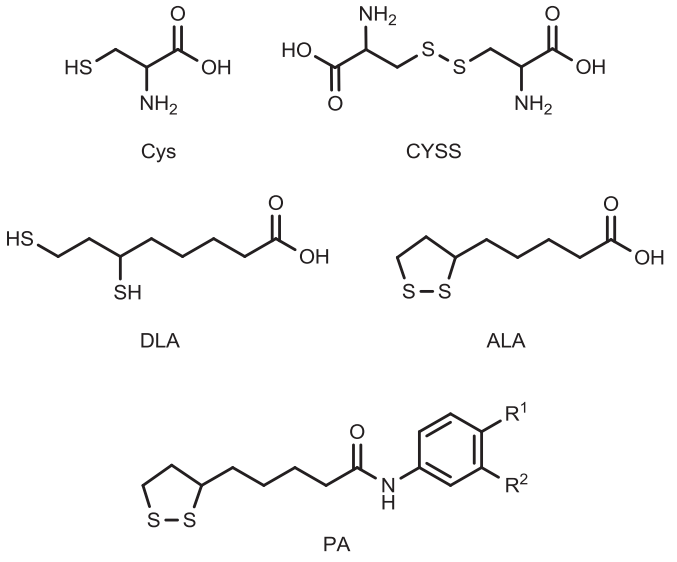

Fig. 3. Selected thiol and disulfide compounds. Cys: cysteine, CYSS: cystine, DLA: dihydrolipoic acid, ALA: α-lipoic acid, PA: N-phenylamide derivatives (R^1, R^2: 1: H, H; 2: NO_2, CF_3; 3: Cl, H; 4: Cl, CF_3; 5: CN, CF_3).

3.3. Tris(2,2′-bipyridine)ruthenium(III)

Tris(2,2′-bipyridine)ruthenium(III) ($[Ru(bipy)_3]^{3+}$) has been widely used for the chemiluminescence and electrochemiluminescence detection of aliphatic amines [39–41], with tertiary substituted amines generally giving the largest responses, followed by secondary and then primary amines [42,43]. Similarly, amino acids such as Pro, Hyp, and pipecolic acid elicit much greater emission intensities than their primary substituted counterparts [43,44]. The reaction is generally accepted to involve oxidation of the amine to form a short-lived cation, and deprotonation of an α-carbon leading to a highly reducing α-aminoalkyl radical that reacts with $[Ru(bipy)_3]^{3+}$ to generate the $[Ru(bipy)_3]^{2+*}$ emitter [17,42,45]. The relationship between analyte structure and emission intensity is therefore dependent on both the formation and the nature of the key radical intermediates. Nevertheless, Noffsinger and Danielson showed an inverse correlation between the logarithm of chemiluminescence intensity and the first vertical ionisation potential of the amine (taken from photoelectron spectroscopy data), which decreases with a greater amine substitution and longer alkyl chain length [42]. In our previous comparison of 20 amino acids with a $[Ru(bipy)_3]^{3+}$ reagent (**F**) prepared by oxidising 0.05 mM $[Ru(bipy)_3]^{2+}$ with 0.2 g/100 mL lead dioxide in 0.02 M sulfuric acid, the response for Pro was two orders of magnitude greater than that of any other analyte [10]. In the current investigation, using similar but not identical instrumental conditions, the selectivity of this reagent towards Pro was not as high (Fig. 4(i)), but it still gave the dominant response of the previously investigated analytes. Hyp and HCYSS (not examined in our previous study) also gave large signals.

A longstanding weakness of $[Ru(bipy)_3]^{3+}$ as a chemiluminescence reagent has been its limited stability in aqueous solution [46], particularly when employing off-line methods of preparation and/or alkaline conditions. To address this issue, we prepared **Reagent G** (by oxidising $[Ru(bipy)_3](ClO_4)_2$ in acetonitrile containing 0.05 M $HClO_4$), which showed no change in chemiluminescence intensity when repeatedly treated with a codeine solution over 48 h [13]. As illustrated by the above discussion of permanganate reagents, subtle changes in reaction conditions can have profound effects upon chemiluminescence intensities, but in this case, **Reagent G** exhibited similar selectivity (Fig. 4(ii)) to that of the more conventional **Reagent F. Reagent G** can be used for extended periods of analysis without the need for recalibration or preparation of fresh reagent solutions and is therefore a far more attractive option for HPLC with post-column chemiluminescence detection. The large signal for HCYSS in Fig. 4(ii) suggests the possibility to also apply this stabilised reagent to the determination of various biologically significant disulfides, although much lower signals were obtained for GSSG and CYSS. The determination of GSH [47] and Cys [48] based on the chemiluminescence reaction with $[Ru(phen)_3]^{2+}$ and either permanganate or cerium(IV) has been reported, but under our conditions, these thiols partially quenched the blank signal. Optimisation of the $[Ru(bipy)_3]^{3+}$ reagent to derive the greatest response for GSH and GSSG under conditions amenable to post-column detection led to a remarkably different selectivity (Fig. 4(iii)), with limits of detection for these two analytes of 7 μM and 1 μM, respectively.

Noffsinger and Danielson noted that the first ionisation potential of simple aliphatic thiols can be assigned to an n-orbital 'lone-pair' electron on the sulfur atom and fall in a region (9.0–9.4 eV), which is similar to that of primary alkyl amines [42]. The corresponding

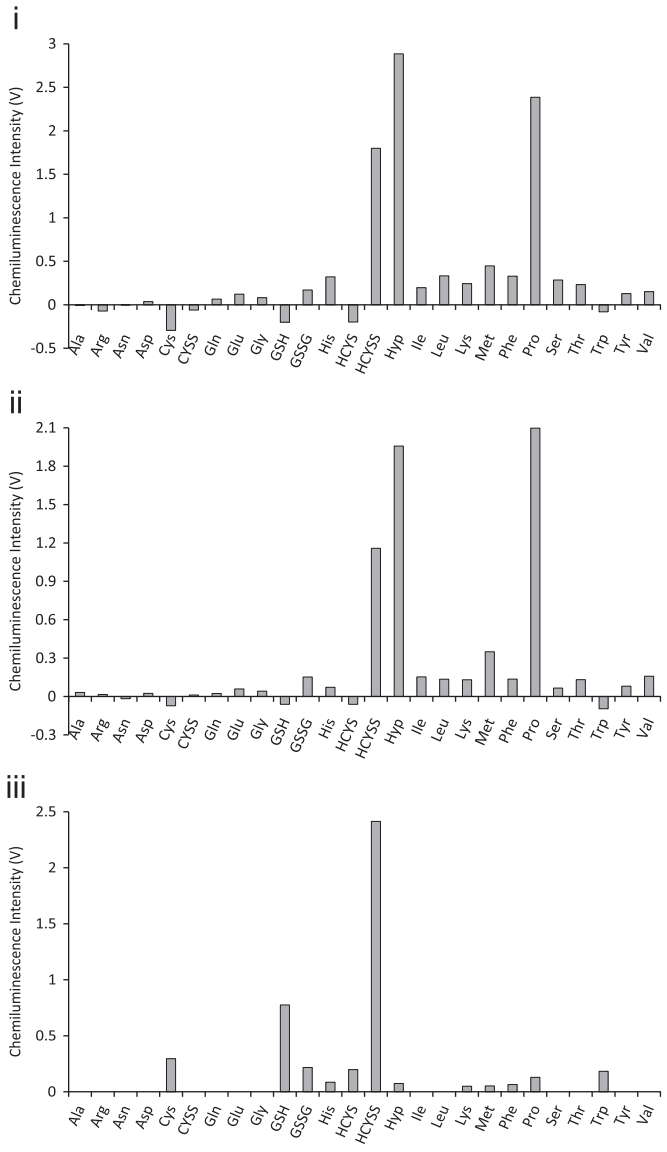

Fig. 4. Relative chemiluminescence intensities with tris(2,2′-bipyridine)ruthenium(III): (i) Reagent F and (ii) Reagent G, where the reagent (0.05 mM) was injected into a carrier stream that merged with a mixture of the analyte (5 μM) and sodium tetraborate buffer (100 mM, pH 10); (iii) Reagent G, where the analyte (10 μM) was injected into a formic acid carrier (pH 2.8) that merged with a phosphate buffer (40 mM, pH 8) stream and then the reagent (1 mM). The average RSD for replicate injections of analytes that gave significant signals under these three sets of conditions was 2.7%, 2.7% and 2.6%, respectively.

thioether and disulfide compounds have lower ionisation potentials [49] and as with the amines [42], the alkyl chain length has a significant influence [49,50], which may in part explain the disparate signals obtained for CYSS and HCYSS, which differ in structure by only one $–CH_2–$ group on each side of the disulfide bond. However, in this case the alkyl chain also distances the disulfide group from the primary amino acid functionality. Perhaps most importantly, a comparison of Fig. 4(ii) and (iii) highlights the importance of reaction conditions on the selectivity of this chemiluminescence detection system.

4. Conclusions

The ability to select chemiluminescence reagents or tune the reaction conditions to target a broad range of compounds or specific chemical classes offers great potential for rapid post-column or post-capillary detection systems. Significant selectivity towards amino acids containing phenol or thiol groups can be derived from the permanganate reagent, whereas the more broadly applicable Mn(IV) reagent presents a useful alternative for the detection of multiple analytes (such as indoles, phenols, thiols, thioethers and disulfides) that would normally require derivatisation and other sample pre-treatment. Although widely used for the detection of secondary amino acids, tris(2,2′-bipyridine)ruthenium(III) may also be useful for the detection of certain biologically important sulfur compounds.

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.2012.06.022.

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