Enhancing the chemiluminescence determination of biologically
and forensically important molecules

by

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I am the author of the thesis entitled:

‘Enhancing the chemiluminescence determination of biologically and forensically important molecules’

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"Do not go where the path may lead, go instead where there is no path and leave a trail."

Ralph Waldo Emerson
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Chemiluminescence, the production of light as the result of a chemical reaction, is an attractive method of detection for a variety of liquid-phase techniques, due to the high selectivity and sensitivity. This thesis encompasses a series of investigations into (i) the design, evaluation and optimisation of novel flow-cells, (ii) improving the sensitivity of several analytically important chemiluminescence reactions via optimisation of reagent chemistry, and (iii) the development of methodologies that exploit these innovations for the determination of biologically significant thiols and disulfides as well as forensically important opiate alkaloids.

Constructing flow-cells for chemiluminescence detection by machining channels into polymer disks has enabled the exploration of new configurations and materials that can improve signal intensity beyond that attainable with the traditional coiled-tubing design. The optimal position of the confluence point was found to be external to the detection zone for several relatively fast chemiluminescence reactions. However, for those with extremely fast kinetics, merging solutions directly in front of the photomultiplier tube provided greater signals. Modifying this design to incorporate central mixing zones with larger widths than the channel reduced the chemiluminescence response. Although the spirally propagating serpentine channel promotes efficient mixing and greater chemiluminescence intensities than a spiral channel, increasing the sharpness of the turns created areas of poor solution flow and decreased the chemiluminescence response. Disks constructed from Teflon impregnated with glass microspheres increased the quantity of light reaching the photodetector, highlighting the importance of surface reflectance.

The chemiluminescence oxidation of organic compounds with acidic potassium permanganate can be catalysed by manganese(II) sulfate or sodium thiosulfate. This
provides greater control of reaction rates, enhanced emission intensities and improved stability when compared to the standard permanganate reagent. This ‘enhanced’ permanganate was used to detect a wide variety of compounds including biomolecules, antioxidants, neurotransmitters, opiate alkaloids and pharmaceuticals. The increased sensitivity arising from this approach resulted in detection limits superior to all previously reported values with acidic potassium permanganate for four model analytes (morphine, synephrine, fenoterol and tyrosine).

The quantification of low-molecular mass thiols and disulfides involved in cellular redox processes is hindered by oxidation or degradation of analytes during conventional sample preparation steps (including deproteinisation and derivatisation). A manganese(IV) colloid was found to provide a simple and sensitive approach for the direct detection of biologically significant thiols and disulfides using both FIA and HPLC methodologies. A mixture of seven thiols and disulfides (cysteine, cystine, homocysteine, homocystine, glutathione (GSH), glutathione disulfide (GSSG) and N-acetylcysteine) in their native forms were separated within twenty minutes. Detection limits for these analytes ranged from $5 \times 10^{-8}$ M to $1 \times 10^{-7}$ M. This procedure was successfully applied to the determination of two key biomarkers of oxidative stress, GSH and GSSG in whole blood taken from twelve human subjects. Samples were simply deproteinised, centrifuged and diluted prior to analysis, using a procedure that was shown to avoid significant auto-oxidation of GSH.

Novel flow-cells with integrated confluence points and reaction channels were evaluated to refine several key design attributes identified throughout the proceeding investigations. Integrating the confluence point into the detection zone facilitated rapid presentation of the reacting solutions to the photodetector. Previously reported
chemiluminescence detectors constructed by machining channels into polymers have almost exclusively been prepared using transparent materials. However, far greater emission intensities were obtained using an opaque white chip with a thin transparent seal, which directed more of the emitted light to the photodetector. This approach also enabled the exploration of channel designs that could not be incorporated into traditional coiled-tubing flow-cells. The combination of these flow-cells (that more efficient mix fast chemiluminescence reactions) with the enhanced permanganate reagent has improved superior limits of detection for a range of analytes.

A rapid HPLC based post-column chemiluminescence screening test for *Papaver somniferum* opiate alkaloids and opiate derivatives in drug seizure samples was developed using the complimentary selectivity of the enhanced acidic potassium permanganate and a stabilised tris(2,2′-bipyridine)ruthenium(III) reagent. The combination of these two stabilised chemiluminescence reagents proved highly compatible with the extended analysis times inherent to HPLC procedures and generated limits of detection comparable to or superior than those previously reported for the *P. somniferum* opiate alkaloids. Furthermore, the addition of an annular frit to the end of the chromatographic column enabled segmentation of the column eluate between three unique modes of detection (UV-absorbance and two chemiluminescence reagents) with only minor losses of sensitivity (less than an order of magnitude) whilst significantly reducing the sample and solvent consumption, waste generation and total analysis time.
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Listed below are the references to publications that have resulted from the work presented in this thesis.


An image from this work was featured on the front cover of the March 2011 issue of Drug Testing and Analysis.


An image from this work was featured on the inside front cover of the March 2011 issue of the Analyst.


An image from this work was featured on front cover of the August 2011 issue of Analytical Chemistry.

CHAPTER ONE

Introduction

- Chemiluminescence
- Chemiluminescence flow-cells
- Biologically important molecules
- Forensically important molecules
- Project aims
1. Chemiluminescence

1.1 General principles

Chemiluminescence is defined as the production of light (ultra-violet, visible or infra-red) as the result of a chemical reaction [1-5]. This reaction typically yields an electronically excited species (C*) which may either emit a photon (direct chemiluminescence; Scheme 1) or donate its energy to a suitable compound (F) which then luminesces (indirect or sensitised chemiluminescence; Scheme 2) [1-4]. This emission process, radiative relaxation of an excited state species to its ground state, is indistinguishable from other forms of luminescence (i.e. fluorescence) [1-4, 6].

\[
A + B \rightarrow C^* + \text{Products} \\
C^* \rightarrow C + \text{Light}
\]

Scheme 1. Direct chemiluminescence

\[
A + B \rightarrow C^* + \text{Products} \\
C^* + F \rightarrow F^* + C \\
F^* \rightarrow F + \text{Light}
\]

Scheme 2. Indirect chemiluminescence

The rate of light generation from these reactions is highly dependent on physical and chemical processes such as solution mixing and reaction kinetics [1-4, 7]. The intensity of the emitted light is proportional to the concentrations of the chemical species involved, and as such, can be used for quantitative analysis [1-4, 7, 8].

There are several inherent advantages of chemiluminescence which have resulted in it becoming an attractive mode of detection for a variety of liquid-phase techniques (such as flow injection analysis, stopped flow analysis, sequential
injection analysis and high performance liquid chromatography) [1-3, 5-8]. Only a relatively low number of chemical reactions are capable of producing chemiluminescence, which results in higher selectivity than other luminescence based techniques [1-4, 7, 8]. Furthermore, the absence of an excitation source results in improved signal-to-noise ratios and greater sensitivity which can lead to superior limits of detection [1-3, 7, 8]. These features, combined with the use of relatively simple instrumentation, have resulted in chemiluminescence detection becoming a widely applied analytical technique [1-3, 5, 7-9].

1.2 Acidic potassium permanganate

In 1917, E.N. Harvey reported the use of acidic potassium permanganate as a chemiluminescence reagent after observing the production of light during the oxidation of a weak solution of pyrogallol [10]. However, the earliest analytical application was not reported until 1975, by Stauff and Jaeschke for the determination of sulphur dioxide [11]. Since Harvey’s initial report, hundreds of papers have been published in scientific literature detailing its analytical applications, including comprehensive reviews by Hindson et al. [12] and Adcock et al. [13].

Despite being used extensively for more than three decades, the light-producing pathway of acidic potassium permanganate has only recently been elucidated [14]. Whilst many researchers erroneously attributed the characteristic red emission to singlet oxygen [13, 15], it has been proven to emanate from a manganese(II) species upon relaxation from an excited state ($^4T_1 \rightarrow ^6A_1$ transition) [13, 16, 17]. Hindson et al. [14] recently demonstrated that the oxidation of an analyte by acidic potassium permanganate [manganese(VII)] generates a radical intermediate and manganese(III) (Figure 1). Subsequent reaction of these species produces an excited state manganese(II) emitter (wavelength of maximum emission 734 ± 5 nm) [14]. The
presence of sodium polyphosphates increases the emission intensity (up to 50-fold) [12, 13] by forming “cage-like” structures around the manganese(II) emitter that prevents deactivation by non-radiative pathways and shifts the wavelength of maximum emission to $689 \pm 5 \text{ nm}$ [13, 14].

![Figure 1](image)

**Figure 1.** Postulated mechanism for permanganate chemiluminescence (adapted from Hindson et al.[14]).

The determination of numerous organic analytes with acidic potassium permanganate has enabled some general trends regarding analyte structure and chemiluminescence intensity to be established. In general, molecules containing phenolic or amine moieties elicit the greatest chemiluminescence responses [12, 13]. Accordingly, several important classes of analytes (antioxidants, biomolecules, illicit drugs and pharmaceuticals) which contain these functional groups have received the most attention to-date [12, 13, 18-27].

### 1.3 Tris(2,2'-bipyridine)ruthenium(III) chemiluminescence

Tris(2,2'-bipyridine)ruthenium(II) $[\text{Ru(bipy)}_3]^{2+}$ was initially synthesised by F.H. Burstall in 1936 [28], but it was not until three decades later that Hercules and Lytle [29] published the first account of chemiluminescence from this complex. They
had observed the production of orange luminescence when a solution of tris(2,2'-bipyridine)ruthenium(III) was reacted with concentrated sodium hydroxide [29]. Comprehensive reviews by Gerardi et al. [30] and Gorman et al. [31] illustrate the numerous analytical applications of this reagent.

Whilst a definitive reaction mechanism for the light-producing pathway has yet to be elucidated, the general detection chemistry is well-established (Figure 2). Common to all analytical applications is the generation of the tris(2,2'-bipyridine)ruthenium(III) reagent $[\text{Ru(bipy)}_3]^{3+}$ via chemical or electrochemical oxidation of $[\text{Ru(bipy)}_3]^{2+}$ [5, 30-33]. Subsequent reaction with a suitable reducing agent (the analyte or analyte oxidation product) results in the formation of an electronically excited state species $[\text{Ru(bipy)}_3]^{2+*}$, which then emits its characteristic orange luminescence (wavelength of maximum emission 610 nm) [5, 30-33].

Figure 2. Generalised mechanism for tris(2,2'-bipyridine)ruthenium chemiluminescence.

The ability to offer a constant, stable supply of the $[\text{Ru(bipy)}_3]^{3+}$ species has seen in situ electrochemistry become the prevalent method of oxidation [30, 31]. However, this technique suffers from downfalls associated with equipment complexity and electrode fouling [30, 31]. An alternative is to employ chemical oxidation using cerium(IV) sulfate or lead dioxide [30, 31]. This approach is rapid
and simple, but does exhibit limited temporal stability, which has to-date significantly impeded the analytical utility of this method of oxidation [1, 5, 30, 31, 33-36]. To overcome this, McDermott et al. [33] recently synthesised [Ru(bipy)_3](ClO_4)_2, which (when dissolved in acetonitrile containing 0.05 M perchloric acid and oxidised with lead dioxide) proved to be exceedingly stable over extended analysis periods (up to 48 hours) and removed the need for constant re-calibration or preparation of fresh reagent solutions.

Tris(2,2′-bipyridine)ruthenium(III) has been used extensively for the chemiluminescence and electrochemiluminescence determination of various analytes containing an amine moiety (in particular amino acids, antioxidants and illicit drugs) [5, 23, 25, 26, 30, 31, 34, 37, 38]. In general, tertiary substituted amines generally elicit the largest response, followed by secondary and then primary [30, 31, 38, 39]. The presence of phenol substituents appears to drastically affect the chemiluminescence signal, with several studies reporting inhibition or quenching of the emission [31, 40, 41].

1.4 Manganese(IV)

Manganese(IV) in its most commonly encountered form - the dark brown manganese dioxide - has been used extensively as an oxidant in many fields of chemistry as detailed in the comprehensive review by Pastor and Pastor [42]. However, the exceedingly poor solubility of manganese(IV) has limited its analytical utility [43].

In 1984, and again in 1993 Jáky et al. reported a method for preparing a ‘soluble’ form of manganese(IV) [44, 45]. This involved the reduction of potassium permanganate with excess sodium formate to yield the characteristic dark brown
manganese dioxide precipitate which was then dissolved into concentrated orthophosphoric acid [44, 45]. Experiments conducted by Jáky et al. using transmission electron microscopy demonstrated that the solution contained small particles less than 2 nm in diameter [44, 45]. Subsequent research conducted by Brown et al. [46] using both scanning and transmission electron microscopies revealed the presence of small uniform nanoparticles (20 nm), which indicated that this form of soluble manganese(IV) was actually a colloidal suspension rather than a solution.

The first report of chemiluminescence from soluble manganese(IV) occurred nearly a decade later when B.J. Hindson [47, 48] used it to determine 25 organic and inorganic analytes. Corrected chemiluminescence spectra revealed that the light emitted from reactions with manganese(IV) had an emission maxima centred at 734 ± 5 nm, which is characteristic of many reactions with manganese-based oxidants [13, 16, 43, 47, 48]. Furthermore, it is suggestive that the red emission emanates from an excited state manganese(II) species [16, 17]. Incorporation of formaldehyde into the reaction system (as an enhancer) significantly improves emission intensity and limits of detection [46-48].

Only a small number of papers concerning the use of manganese(IV) as a chemiluminescence reagent have been published [43]. From these, no general trends with respect to molecular structure and intensity have emerged, but it is apparent that the reagent exhibits markedly different selectivity to that of acidic potassium permanganate [43].
1.5 Instrumentation

1.5.1 Flow injection analysis

Flow injection analysis (FIA) was first introduced in 1975 by Ruzicka and Hansen [49] and Stewart et al. [50] as a rapid method of sample handling for laboratory analysis. This simple dispersion technique allows reactions to be performed by injecting a precise aliquot of sample into a continuously flowing carrier stream, which subsequently merges with a reagent [2, 4, 7, 8, 49-53]. Three decades ago, Rule and Seitz published the first account of FIA with chemiluminescence detection, in which copper was used to catalyse the luminol-hydrogen peroxide reaction [54].

Many chemiluminescence reactions occur very rapidly, and due to the transient nature of the emission, a significant advantage of using FIA methodology is the ability to reproducibly merge two reacting solutions as close as possible to the detector [2, 4, 7, 8, 52, 53]. To ensure that the maximum amount of radiation is captured by the detection system (usually a photomultiplier tube) factors such as the reaction kinetics, volume between the confluence and observation points, the geometry of the flow-cell and the flow rate all need to be carefully optimised [2, 7, 8, 52, 53].

Since Rule and Seitz’s initial report [54], hundreds of papers have been published in scientific literature examining the use of FIA to: (i) explore the fundamental chemistry behind chemiluminescence reactions, (ii) optimise post-column reaction conditions for liquid chromatography and (iii) quantify analytes in simple matrices as detailed in the comprehensive review by Fletcher et al. [8].

There are however, some limitations associated with using chemiluminescence as a detection method for FIA [2, 7, 52]. The addition of reagents and modifications to
the instrumental setup increases the overall complexity of the manifold [2, 7, 52]. This can lead to lower reproducibility, particularly if different flow rates are used. Furthermore, due to the simple instrumentation and the lack of a separative component in traditional manifolds, FIA does not have the selectivity to determine multiple analytes within complex biological matrices [2, 7, 52].

1.5.2 Sequential injection analysis

Sequential injection analysis (SIA) was developed in 1990 by Ruzicka and Marshall to address some of the aforementioned limitations of FIA and the need for a more powerful and versatile flow-based sample-handling methodology [55]. Unlike FIA where a sample is injected into a continuously flowing carrier stream and a reagent is subsequently merged on-route to the flow-cell [2, 4, 7, 8, 49-53], SIA utilises one flowing stream under the control of a bidirectional pump [55-59]. This pump initially aspirates the carrier solution followed by defined aliquots of sample and reagent; each introduced via the action of a computer controlled multi-position valve [55-59]. This sequential aspiration results in a defined series of sample and reagent zones which are directed towards a holding coil [55-59]. The direction of flow is then reversed and the sample and reagent zones are propelled towards the flow-cell creating product zones from the overlapping regions of the sample and reagent(s) [55-59]. It is these zones that generate the radiation detected by the photomultiplier tube [56-58] (Figure 3).
Figure 3. Schematic diagram illustrating: (i and ii) the pump operating in the reverse mode to aspirate and sequentially stack plugs of reagent (R) and sample (S) and (iii) reversal of flow resulting in the formation of a zone of detectable product (P). Arrows indicate the direction of flow (adapted from [56]).

Owing to the use of robust syringe or Milligat pumps and computer control, the advantages of SIA over the more traditional FIA are; (i) the capacity to stack, in any order, precisely defined zones of sample and reagent that are mixed to yield detectable products, (ii) lower reagent consumption, and (iii) production of far less waste [2, 55-58]. A disadvantage of SIA is the lower sample throughput, as the indispensable step of stacking the sample and reagents in the holding coil prior to reversing the direction of flow results in longer run times than FIA [2, 55-58].

1.5.3 High performance liquid chromatography

Reversed-phase high performance liquid chromatography (HPLC) is a powerful method of identifying, quantifying and purifying specific compounds in complex matrices (i.e. process samples, pharmaceuticals and physiological fluids) [60-62]. The separation of each component in a given sample is attributable to hydrophobic interactions and repulsive forces occurring between a moderately polar mobile phase (typically water containing an organic modifier), the sample, and a nonpolar stationary phase (consisting of a hydrophobic ligand chemically bound to a
particulate or solid support) [60-62]. As the components within a sample are separated, they elute off the column and into a detector, typically UV-absorption or fluorescence, where their individual retention times are recorded in the form of a chromatogram [60-62].

Coupling HPLC with chemiluminescence detection involves merging a flowing stream of the chemiluminescence reagent(s) (via the action of a peristaltic or syringe pump) with the column eluate at a confluence point located prior to the point of detection [5, 6, 9, 52]. Post-column chemiluminescence introduces selectivity that is normally unattainable with FIA, and offers improved sensitivity when compared to the aforementioned detection techniques [5, 6, 9, 52]. Compromises between the chemical parameters required for an efficient chromatographic separation and sensitive post-column detection are usually required, as chemiluminescence emissions are extremely susceptible to changes in pH, flow-rate and the amount and type of organic modifier in the mobile phase [5, 6, 9, 52].

1.5.4 Stopped flow analysis

In flow-based methodologies such as FIA, SIA or HPLC, the analyte, carrier and reagent(s) are propelled in streams and subsequently merged to initiate the chemiluminescence reaction close to or within the flow-cell [2, 4, 7, 8, 49-53]. This results in only a small portion of the total emission being captured by the photodetector [63, 64] (Figure 4i). In stopped flow analysis, discrete quantities of the reagent(s), carrier and analyte are propelled by a syringe pump into a detection chamber or flow-cell, where they are held for a set period of time [65-68]. This enables the entire chemiluminescence intensity versus time profile to be monitored [68] (Figure 4ii). Generally, the temporal distribution of the signal is proportional to the analyte concentration and can provide information about the reaction kinetics.
which may be exploited to aid in the optimisation of reaction and manifold conditions for flow-based methodologies [68]. Furthermore, in cases where the chemiluminescence kinetics are distinct for two or more compounds, the signals resulting from mixtures of analytes can be mathematically derived to provide enhanced selectivity without a separation step [67-69].

![Chemiluminescence Intensity vs Time](image)

**Figure 4.** Schematic of a typical chemiluminescence peak where (i) depicts the portion of the emission captured by the detector using FIA methodology and (ii) depicts the portion of the emission captured by the detector with stopped flow analysis.

## 2. Chemiluminescence flow-cells

Whilst each of the aforementioned analytical techniques is unique with regard to the manipulation, mixing and transportation of analytes and reagents to the point of detection, inherent to each is the presence of a flow-cell which retains the reacting solution in view of a photomultiplier tube [2, 4, 6, 7, 52, 70]. The design and geometry of the flow-cell is crucial; it needs to be transparent, have appropriate channel dimensions to ensure efficient mixing and maximise sensitivity, and be inert to the chemical reaction being employed [2, 4, 6, 7, 52, 70].

Coiled-tubing flow-cells were first introduced for chemiluminescence detection in FIA by Rule and Seitz in 1979 [54]. The cells were simple to construct, compatible
with photodetector geometry, and enabled collection of the emitted light during the first few seconds of the reaction [6, 53, 54, 71, 72]. More than three decades later the most commonly reported flow-cell configuration for flow analysis based techniques still consists of a coil of glass or polymer tubing (normally 0.5-1.0 mm i.d.) mounted against a photomultiplier tube within a light-tight container [2, 4, 6, 7, 52, 53, 70]. Solutions merge at a T- or Y-shaped junction shortly before entering the coil (Figure 5). However this simple design has several serious limitations; (i) the walls of the tubing are curved, and therefore most of the surface is not flat against the photodetector window, (ii) polymer tubing is often translucent rather than totally transparent, (iii) the geometry of the flow-cell is hindered by the limited flexibility of polymer or glass tubing, (iv) mixing is initiated before the reacting mixture enters the coil and (v) the internal diameter of the tubing is limited by availability [7, 73].

![Figure 5. Schematic of a traditional chemiluminescence flow-cell used in various flow analysis based techniques [53].](image)

A variety of alternative chemiluminescence detection cells have been described, yet to-date none of these designs have been adopted for routine analysis [74-83]. In the fountain flow-cell [83], the reacting mixture enters the centre of an open, shallow cylindrical space and drains into a ring-shaped well around the edge, which contains the outlet hole. This configuration allows a greater volume of solution to be in contact with a flat surface facing the photodetector. Another design, referred to as the
bundle cell [81], consists of a bundle of Teflon tubing packed into a plastic cuvette. This configuration was found to be 50% more efficient than a coil composed of the same tubing [81]. However, the first turn of the coil used in that particular study had a diameter of 1 cm, leaving a relatively large empty space in the centre of the coil compared to similar flow-cells constructed by other researchers. These studies help to highlight the importance of optimising flow-cell design and geometry to maximise mixing efficiency and detection of the emitted light.

3. Biologically important molecules

3.1 Low-molecular mass thiols and disulfides

Low-molecular mass thiols such as homocysteine, cysteine and glutathione, are critical physiological components endogenous to all biological tissues and fluids [84-88]. These thiols are mainly present in their reduced forms which can be converted to the corresponding disulfide (cystine, homocystine and glutathione disulfide) under conditions of oxidative or nitrosative stress [84-88].

Homocysteine is an endogenous thiol-containing amino acid synthesised in the liver as an intermediate of the methionine cycle (Figure 6) [84, 86, 89, 90]. Under normal conditions, intracellular concentrations of homocysteine are kept relatively low as the result of (i) remethylation back to methionine using the vitamin B12-dependent methionine synthetase and/or (ii) conversion to cysteine via the trans-sulfuration pathway [84, 86, 89-92]. Elevated concentrations of homocysteine in physiological fluids are considered to be an important risk factor or marker for several diseases, and as such interest in the determination of this biologically significant thiol has rapidly grown during the last decade [84, 89, 90, 93-95].
Cysteine can be derived \textit{in vivo} from two independent metabolic pathways [84, 86, 89, 91, 92, 96]. In the trans-sulfuration pathway, homocysteine is converted to a cystathionine intermediate which is then metabolised to form cysteine [84, 86, 89, 91, 92, 96] (Figure 6). In the \(\gamma\)-glutamyl cycle, the breakdown of glutathione by \(\gamma\)-glutamyl transpeptidase produces a cysteinylglycine fragment which is subsequently cleaved by membrane-bound dipeptidases to form cysteine and glycine (Figure 6) [85, 86, 96]. A variety of important cellular functions, including protein synthesis, detoxification, and metabolic processes are associated with this endogenous thiol [84-86, 89, 91, 92, 96].

The tripeptide glutathione (\(\text{L-}\gamma\)-glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol produced in mammalian organs, tissues and cells [84-89, 96]. Glutathione (GSH) is synthesised \textit{via} two adenosine triphosphate (ATP) dependent steps (Figure 6) [85, 86, 89, 96]. In the rate limiting step, the dipeptide \(\gamma\)-glutamylcysteine is synthesised \textit{via} condensation of cysteine and glutamate by the enzyme \(\gamma\)-glutamylcysteine synthetase [85, 86, 89, 96]. Glycine is then added to the C-terminal of \(\gamma\)-glutamylcysteine \textit{via} the action of glutathione synthetase to form GSH [85, 86, 89, 96]. The presence of a \(\gamma\)-glutamyl linkage and thiol moiety enables GSH to participate (either directly or indirectly) in many biological processes [85, 86, 89, 96]. These include protein and DNA synthesis, regulation of enzyme activity, detoxification of electrophilic xenobiotics, maintenance of sulfhydryl groups in proteins, protection of cell membranes from oxidative damage and antioxidant defence [84-86, 89, 96-99].
Figure 6. Schematic representation of the methionine cycle, trans-sulfuration pathway and γ-glutamyl cycle (adapted from [85, 86, 89]).

3.1.1 GSH and oxidative stress

Reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) are produced as a result of normal cellular metabolic processes such as respiration and photosynthesis [100-102]. These species play a dual role within biological systems, having both deleterious and beneficial effects [88, 100-105]. At low/moderate
concentrations, ROS/RNS play crucial roles in various physiological processes including: defence against infectious agents, enhancement of signal transduction from various membranes, sensing of oxygen tension, induction of mitogenic responses and regulation of redox homeostasis [88, 100-105]. The deleterious effects occur when there is an over production of ROS/RNS, coupled with a deficiency of enzymatic and non-enzymatic antioxidants [88, 100-105]. This causes damage to and/or inhibits the normal functioning of cell structures, including lipids and membranes, proteins, and DNA and is referred to as oxidative stress or nitrosative stress [88, 100-105].

In protecting against oxidative stress and free-radical-mediated cell injury, GSH can donate a reducing equivalent, resulting in the formation of glutathione disulfide (GSSG) [85, 88, 97-100]. Regeneration of GSH from GSSG is mediated by the widely distributed enzyme glutathione reductase in a nicotinamide adenine dinucleotide phosphate (NADPH) dependant reaction [85, 88, 97-100] (Figure 6). Typically, the molar ratio of GSH/GSSG in eukaryotic cells is between 10:1 and 1000:1; however, under conditions of oxidative stress, this ratio decreases [85, 88, 97-100]. As oxidation of the free thiol to the corresponding disulfide is one of the earliest responses to an overproduction of reactive oxygen species, the accurate and interference-free measurement of the GSH/GSSG ratio offers great potential for diagnosing and monitoring pathological and physiological conditions related to oxidative stress [84, 85, 87, 88, 97-100, 106-110].

3.2 Determination of biologically important thiols and disulfides

Since the isolation of GSH from yeast and animal tissues by Hopkins [111] in 1921, numerous analytical methodologies have been developed to quantify low-molecular mass thiols and disulfides in biological fluids due to their importance
in diagnosing and monitoring pathophysiological conditions associated with oxidative stress [85, 97, 99, 112, 113].

However, several significant analytical challenges associated with the accurate measurement of thiols and disulfides have emerged [84, 85, 87, 107, 109, 110, 114, 115]. Due to the lack of strong chromophores or fluorophores, derivatisation of these analytes is essential for techniques employing spectrophotometric or spectrofluorimetric detection [84, 85, 87, 107, 109, 110, 114, 115].

Thiols are usually derivatised with either a colorimetric (such as Ellman’s or Sanger’s) or fluorogenic reagent(s) (such as bimanes, malimides, halides, halogenosulfonylbenzofurazans, or ortho-phthaldehyde) prior to chromatographic separation and detection [84, 85, 87, 107, 109, 110, 114, 115]. Disulfides then need to be reduced to their corresponding thiols via the use of reagents such as dithiothreitol, 2-mercaptoethanol or tris(2-carboxyethyl)phosphine prior to the aforementioned derivatisation, separation and detection procedures [84, 85, 87, 107, 109, 110, 114, 115]. The disulfide concentration is then calculated from the difference between the measurements obtained in the two separate analytical steps. This approach is time consuming and vulnerable to several major sources of error [87, 115], the most important of which is the facile auto-oxidation of thiols under the alkaline conditions required for derivatisation and disulfide bond reduction [84, 85, 87, 106-109, 116, 117].

Physiological fluids, particularly whole blood, plasma and tissue samples contain proteinaceous materials that need to be removed prior to analysis [84, 85, 87, 106, 107, 110]. Typically, acidification of the physiological fluid (with either 5-sulfosalicylic, metaphosphoric, perchloric, trichloroacetic or trifluoroacetic acids) in combination with centrifugation provides a simple method of inducing protein
precipitation and yields a clear protein-free supernatant suitable for analysis [84, 85, 87, 106, 107, 110]. Disulfides are present in biological samples at much lower concentrations than thiols, and even a small degree of auto-oxidation or incomplete removal of proteins during these sample preparation procedures can lead to their considerable overestimation [84, 85, 87, 106-109, 116, 117].

Therefore, the determination of these analytes should ideally combine minimal sample handling (under conditions that prevent auto-oxidation) with direct detection of all target analytes in a single chromatographic separation. Thiols and disulfides have previously been detected without derivatisation using electrochemistry, mass spectrometry or fluorescence quenching [84, 87, 107, 110, 116-119]. Whilst these modes of detection offer advantages over more popular derivatisation techniques in terms of procedural simplicity, their application is limited by equipment complexity, cost, electrode stability and/or analysis time [108, 110, 120, 121].

Several reviews have highlighted the selectivity and sensitivity of chemiluminescence detection when applied to a variety of liquid-phase techniques such as FIA, SIA and HPLC [2, 4, 5, 8, 9, 12, 13, 30, 31, 122]. The inherent selectivity of this technique arises as a result of the limited number of species capable of emitting light [1]. This effectively eliminates interferences from non-reacting species present in complex matrices (such as biological fluids) that are typically observed with other detection methods.

Our research group recently reported the determination of GSH in cultured skeletal muscle cells with acidic potassium permanganate chemiluminescence detection following a rapid chromatographic separation [18]. However, like other previously reported chemiluminescence-based detection systems for thiols, this reagent was not suitable to detect disulfides [123, 124]. Therefore quantification of GSSG required
thiol blocking and disulfide bond reduction steps prior to a second separative step [18].

These issues point to the need for an approach that (i) prevents or minimises auto-oxidation during sample preparation, (ii) removes the requirement of a time-consuming derivatisation step and (iii) allows for the direct detection of biologically significant thiols and disulfides with simple instrumentation following a single chromatographic separation.

4. Forensically important molecules

4.1 Opiate alkaloids

Opium is a natural product obtained from the unripened seed pods of Papaver somniferum, or the opium poppy [125-130]. Small incisions in the skin of an unripened seed pod are used to obtain the milky opium exudate which consists of proteins, sugars, lipids and approximately thirty alkaloids [125-130].

Despite opium and the opium poppy being used recreationally and medicinally for centuries, the active ingredient responsible for physiological effects such as analgesia, sedation and respiratory depression [127, 131] was not identified until the early nineteenth century when F.H. Sertturner reported the isolation of morphine [126, 132]. Since this time, many more P. somniferum alkaloids have been identified, and in general they fall into two distinct categories based upon their chemical structure; phenanthrene alkaloids (such as morphine, codeine, thebaine and oripavine) and isoquinoline alkaloids (such as papaverine and noscapine) [125, 126, 128]. The relative concentrations of these alkaloids vary substantially from poppy to poppy and are highly dependent on factors such as climate, soil fertility, altitude, available moisture, and the age of the plant [125, 126, 128].
The commercial sale of preparations containing morphine, as well as the invention of the hypodermic syringe saw a rapid increase in the popularity of the drug, and by the late nineteenth century, efforts were being made to find a non-addictive alternative [133]. In 1874, whilst working at Saint Mary’s Hospital Medical School, C.R. Alder Wright acetylated carbon 3 and 6 of morphine’s phenanthrene backbone to form the semi-synthetic derivative 3,6-diacetylmorphine or heroin [125, 133-135]. Early reports suggested that when compared to morphine, heroin was more effective at depressing the respiratory system and had a much weaker narcotic action [135, 136]. As a result of these studies, heroin was introduced into the commercial market as a cough suppressant and treatment for tuberculosis [133, 135]. After addiction to these medications rapidly increased, it was discovered that by simply acetylation of the phenolic and hydroxyl moieties of the phenanthrene backbone, Wright had created a narcotic even more powerful and addictive than its parent compound [130, 133, 135]. Despite products containing heroin being quickly withdrawn from commercial sale and the establishment of the International Convention on Narcotics in 1924, the 1950’s saw a significant rise in the illegal manufacture and misuse of the drug [133, 135].

The presence of acetyl groups on the heroin molecule significantly increases the lipid solubility of the drug, allowing it to easily cross the blood-brain barrier [127, 131, 137]. Heroin is considered to be a prodrug; a drug that is rapidly converted into the active therapeutic form after absorption [131, 138]. Once in the brain, it is rapidly deacetylated to its pharmacologically active metabolites morphine and 6-monoacetylmorphine [127, 131, 137]. These metabolites interact with numerous \( \mu \)-opioid receptors located in the central nervous system to lessen or remove the sensation of pain via disruption of nerve impulses [127, 131, 137]. Further metabolism results in morphine being conjugated to a glucuronide to render it
soluble for excretion from the body [127, 137]. This glucuronide is a polar molecule and a derivative of an acidic compound made from glucose [127, 137]. Once conjugated to morphine it is detectable in urine for 2-4 days after administration of the drug [127, 137].

Each year the pharmaceutical industry extracts and purifies hundreds of tonnes of opiate alkaloids from *P. somniferum* for medicinal use [139]. Therefore, the accurate measurement of these species is of great importance when monitoring industrial-scale extractions of alkaloids from opium poppies and establishing the alkaloidal content of these process extracts and pharmaceutical formulations [126, 139]. Furthermore, the on-going illicit production of heroin has resulted in it being considered as one of the most significantly abused narcotics [128, 129]. This has created the need to identify and/or quantify the drug (and its precursors or metabolites) in suspected drug seizure samples, biological fluids, hair samples and the larvae of insects found on or near the bodies of drug overdose victims [25, 26, 125, 130, 140].

### 4.2 Determination of opiate alkaloids

Numerous analytical methodologies exist for the identification and quantification of opiate alkaloids [125, 130, 141-144]. Screening tests are often utilised to obtain rapid preliminary identification of certain illicit substances or classes of drugs prior to confirmatory testing with more specific instrumental methods [125, 128, 129, 145, 146].

The most commonly used screening method for opiates and opiate derivatives involves mixing seizure samples with various reagents, such as Mandelin’s, Marquis or Mecke’s, and monitoring the resultant colour change [125, 128, 129, 145, 146].
These spot tests are usually selective for a particular class of drug (i.e. opiates), but are not selective for specific drugs (i.e. heroin), so a series of tests is usually performed. Greater specificity can be obtained by performing microcrystalline precipitation tests [125, 128, 129, 145]. However, the presence of cutting agents and diluents can result in significant alterations to the crystal structure which can impede identification [125, 128, 129, 145].

Characterisation and quantification of single or multiple analytes in complex matrices (such as drug seizure samples, pharmaceutical process streams and bodily fluids) is generally undertaken with either; (i) gas chromatography coupled to flame ionisation or mass spectrometric detection, or (ii) HPLC and/or capillary electrophoresis (CE) with UV-absorbance, fluorescence, electrochemical or mass spectrometric detection [125, 130, 141-144].

As previously mentioned, chemiluminescence offers highly sensitive and selective detection for species capable of emitting light, effectively eliminating interferences from non-reacting species present in complex matrices that are typically observed with other detection methods [1, 2]. As described in several reviews, the chemiluminescence detection of opiate alkaloids and their derivatives has been largely dominated by two reagents: acidic potassium permanganate and tris(2,2'-bipyridine) ruthenium(III) [12, 13, 25, 26, 30, 31].

The first report of chemiluminescence from the reaction of illicit drugs and pharmaceuticals (antibiotics, monoamine oxidase inhibitors and opiate alkaloids) with acidic potassium permanganate was published in 1986 by Abbott and Townshend [147]. Whilst numerous analytes have been examined since this time, very few can be detected at the exceedingly low concentrations reported for morphine and other P. somniferum opiates [13]. A range of narcotic analgesics
(including opiate alkaloids) were subsequently screened with acidic potassium permanganate by Abbott and co-workers [75] and Barnett and co-workers [148]. Analytes containing a phenolic moiety at carbon 3 on the phenanthrene backbone and a furan bridge between carbons 4 and 5 were found to produce superior chemiluminescence emissions when compared to other analytes investigated [75, 148]. Substitution of the functional group at carbon 3 had a dramatic influence on chemiluminescence intensity, with opiates containing a methoxy moiety (codeine and thebain) generating responses 0.5-2% that of morphine [148].

Our research group has previously reported several applications of acidic potassium permanganate chemiluminescence for the selective and sensitive determination of phenolic morphinan alkaloids (morphine and oripavine) in process samples using FIA, SIA, HPLC and CE [27, 148-154].

The first chemiluminescence determination of non-phenolic opiate alkaloids (codeine, heroin and dextromethorphan) with electrochemically oxidised tris(2,2'-bipyridine)ruthenium(III) was reported by Greenway and co-workers [155] in 1995. Subsequent research by Barnett and co-workers [156] employed chemically oxidised tris(2,2'-bipyridine)ruthenium(III) to determine non-phenolic morphinan alkaloids in P. somniferum process extracts. A comprehensive examination of several phenolic and non-phenolic opiate alkaloids and their derivatives revealed that emission intensities were highly dependent on reaction pH and molecular structure, with phenolic moieties and quaternary nitrogens quenching the response [41]. Conversion of the phenolic moiety to a methoxy (codeine, 6-methoxycodine and thebaine) or an acetyl group (heroin) evoked intense chemiluminescence emissions with the tris(2,2'-bipyridine)ruthenium(III) reagent [41]. Our research group has reported the selective determination of several non-phenolic morphinan alkaloids
using tris(2,2’-bipyridine)ruthenium(III) chemiluminescence detection coupled to FIA, SIA, HPLC and CE [27, 33, 152-154, 156-158].

The complimentary selectivity of these reagents for phenolic and non-phenolic morphinan alkaloids served as the basis of a rapid screening test for heroin in drug seizure samples developed by Agg and co-workers [158] (Figure 7). Heroin produces an intense chemiluminescence response with tris(2,2’-bipyridine)ruthenium(III) and a relatively weak response with acidic potassium permanganate. When the seizure sample is mixed with a small amount of a concentrated base, heroin is rapidly converted into its metabolites, morphine and 6-monoacetylmorphine, which elicit an intense response with acidic potassium permanganate and a negligible response with tris(2,2’-bipyridine)ruthenium(III) [158]. This methodology has been successfully applied to several drug seizure samples using both FIA and SIA approaches [27, 158]. However, the absence of a separative component in the two instrumental approaches has, to date, prevented the identification of various opiates and/or alkaloidal impurities within these seizure samples.

![Figure 7. Concept for the rapid chemiluminescence screening test for heroin (adapted from Agg et al. [158]).](image-url)
5. Project aims

This thesis describes a systematic approach to the design, evaluation and optimisation of novel flow-cells for chemiluminescence detection in both flow analysis and HPLC. Various approaches were employed for improving the sensitivity of several analytically important reactions utilised for the determination of biologically significant thiols and disulfides as well as forensically important opiate alkaloids.
Solution mixing in flow-cells for chemiluminescence detection

- Introduction
- Experimental
- Results and discussion
- Conclusion
1. Introduction

Chemiluminescence detection has been used extensively in procedures based on flow injection analysis (FIA) and sequential injection analysis (SIA) methodology [2, 8, 9, 57, 159], due in part to the excellent sensitivity and wide calibration ranges that have been obtained for diverse classes of analytes. Moreover, the instrumentation is simple, essentially comprising a reaction vessel or conduit with a transparent surface, mounted against a photodetector. The most commonly used configuration consists of a coil of glass or polymer tubing (normally 0.5-1.0 mm i.d.) mounted against a photomultiplier tube within a light-tight container. Solutions merge at a T- or Y-shaped junction shortly before entering the coil.

The emission of photons from a chemiluminescence reaction is transient and occurs at a rate that is dependent on both the kinetics of the chemical reaction and the physical processes of solution mixing [8, 71]. For the greatest sensitivity, the instrumental manifold and the flow-cell should be configured to maximise the emission and detection of light when the reacting mixture passes through the cell [2]. For relatively fast chemiluminescence reactions, the analyte and reagent solutions should merge at (or as close to) the point of detection. In addition, the dead volume should be minimised to ensure rapid rinsing of the cell between analyses.

Etching or machining channels into glass/polymer materials and sealing the channels with a transparent surface provides a convenient and reproducible alternative that enables exploration of new materials or configurations to enhance solution mixing, maximising the transfer of light to the photodetector [73, 160]. This approach is also utilised to create chemiluminescence reaction zones within microfluidic chips [161-164]. Previously reported configurations include linear channels [165, 166], spirals (that mimic the traditional coil of tubing approach) [73,
160, 167-169], and ‘meandering’ or ‘serpentine’ designs comprising a linearly propagating series of reversing turns [163, 170]. Spiral or meandering channels consisting of linear segments between right-angled turns have also been reported [161, 162, 164, 171, 172].

This chapter describes a novel chemiluminescence detector designed by Global FIA to maximise both the generation and transmission of light from chemiluminescence reactions. The key component of this new detector is a thin Teflon disk with channels machined into one side. The Teflon disk is placed against a sapphire window to form a flow channel with a flat transparent seal allowing efficient transmission of light to the photomultiplier tube. The channel configuration can be conveniently modified by replacing the Teflon disk, and as such, several novel designs have been explored and compared with a wide range of analytically useful chemiluminescence reactions, such as: morphine with acidic potassium permanganate; vanilmandelic acid with luminol and hexacyanoferrate(III); codeine with tris(2,2’-bipyridine)ruthenium(III); and arginine, urea or ammonium with hypobromite.
2. Experimental

2.1 Chemicals and reagents

Deionised water (Continental Water Systems, Victoria, Australia) and analytical grade reagents were used unless otherwise stated. Chemicals were obtained from the following sources: L-arginine, luminol, sodium polyphosphate (+80 mesh) and vanilmandelic acid from Sigma-Aldrich (New South Wales, Australia); hydrogen peroxide (3%) and potassium permanganate from Chem-Supply (South Australia, Australia); bromine, lead dioxide, potassium ferricyanide, sodium hydroxide and urea from Ajax Finechem (New South Wales, Australia); codeine and morphine from GlaxoSmithKline (Victoria, Australia); sulfuric acid from Merck (Victoria, Australia); ammonium chloride from BDH (Poole, England) and tris(2,2'-bipyridine) ruthenium(II) dichloride hexahydrate from Strem Chemicals (Minnesota, USA).

Stock solutions \((1 \times 10^{-3} \text{ M})\) of morphine and codeine were prepared in acidified deionised water and sonicated to aid dissolution. Vanilmandelic acid \((1 \times 10^{-3} \text{ M})\) was dissolved in aqueous sodium hydroxide \((0.1 \text{ M})\), whilst ammonium chloride, arginine and urea \((1 \times 10^{-3} \text{ M})\) were prepared daily in deionised water. When required, the analytes were diluted into deionised water.

The acidic potassium permanganate reagent \((1 \times 10^{-3} \text{ M})\) was prepared by dissolution of potassium permanganate in a 1% \((\text{m/v})\) sodium polyphosphate solution and adjusted to pH 2.5 with sulfuric acid.

The luminol reagent \((3.5 \times 10^{-6} \text{ M})\) was prepared by dissolution of the solid in deionised water, following the addition of a small volume of aqueous sodium
hydroxide (1.0 M). Potassium hexacyanoferrate(III) (potassium ferricyanide, $3 \times 10^{-4}$ M) was prepared in sodium hydroxide (0.35 M).

To obtain intense luminol chemiluminescence suitable for visual examination, the luminol reagent was prepared by dissolution of luminol ($1.1 \times 10^{-3}$ M) in sodium hydroxide (0.1 M), whilst potassium hexacyanoferrate(III) (potassium ferricyanide, $3 \times 10^{-2}$ M) was prepared in deionised water containing 4% (v/v) hydrogen peroxide [173].

The tris(2,2'-bipyridine)ruthenium(III) reagent ($1 \times 10^{-3}$ M) was prepared by dissolving tris(2,2'-bipyridine)ruthenium(II) dichloride hexahydrate in dilute sulfuric acid (0.05 M). The ruthenium(III) species was obtained by adding lead dioxide (0.2 g per 100 mL) to the reagent solution and stirring for 5 minutes. Following the characteristic change in colour from bright orange to emerald green, excess oxidant was filtered (0.45 μm, Millipore MillexHN Nylon) from the solution prior to analysis.

Hypobromite (0.08 M) was prepared daily by disproportionation of bromine in ice cold sodium hydroxide (0.4 M). To prevent photodegradation of the reagent, the Schott bottle was covered in aluminium foil.

2.2 Flow injection analysis

The FIA manifold was constructed from a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Victoria, Australia) with bridged PVC pump tubing (1.02 mm i.d.; DKSH, Queensland, Australia), black manifold tubing (0.76 mm i.d., Global FIA, Washington, USA) and six-port injection valve (Vici 04 W-0192L, Valco Instruments, Houston, Texas, USA). The conventional and novel flow-cells were mounted against the window of an extended range photomultiplier module.
(Electron Tubes P30A-05, ETP, NSW, Australia) in light-tight housing. The output from the photomultiplier captured by a chart recorder (YEW type 3066, Yokogawa Hokushin Electric, Tokyo, Japan) or an ‘e-corder 410’ data acquisition system (eDAQ, NSW, Australia).

For acidic potassium permanganate and hypobromite measurements, the analyte standards were injected (70 μL) into a deionised water carrier stream that merged with the chemiluminescence reagent shortly before, or within the flow-cell (Figure 8). In the case of the ruthenium(III) complex, the reagent was injected into a dilute sulfuric acid (0.05 M) carrier stream that merged with the analyte solution shortly before or within the flow-cell. Solution flow rates were optimised over the range 0.9 mL/min to 3.5 mL/min per line, for each chemiluminescence reaction.

![Figure 8. Schematic of the FIA manifold](image)

2.3 Sequential injection analysis

The SIA manifold was constructed from a milliGAT pump (model CP-DSM-GF, Global FIA), ten-port multi-position valve (model C25Z, Valco) and black manifold tubing (0.76 mm i.d., Global FIA). The conventional and novel flow-cells were mounted against the window of an extended range photomultiplier module (Electron Tubes P30A-05, ETP, NSW, Australia) in light-tight housing. A desktop computer equipped with data acquisition board (LabJack U12, National Instruments, Victoria,
Australia) and LabView Software (Version 8.0, National Instruments) was used to automate the instrument and acquire experimental data.

2.3.1 Conventional SIA mode

This automated system was programmed to aspirate and sequentially stack the carrier (line 1, 2050 μL, 167 μL/s), analyte (line 2, 500 μL, 20 μL/s), and reagent (line 3, 150 μL, 20 μL/s) into a holding coil (PTFE tubing, DKSH, 2.7 m × 0.8 mm i.d.). The direction of flow was then reversed to propel the reacting mixture (167 μL/s) through the detector (Figure 9).

![Figure 9. Schematic of the conventional SIA manifold](image)

2.3.2 Direct aspiration mode

The detector was placed in-line between the pump and multi-position valve, so that the solutions were sequentially aspirated directly through the flow-cell without the reversal of solution flow (Figure 10). This results in minimal mixing of solutions prior to entry into the chemiluminescence detector. This automated system was programmed to repeatedly aspirate 150 μL of the reagent (valve position 1; 167 μL/s), 1500 μL of the analyte standard (position 2; 167 μL/s), and 1000 μL of the carrier solution (position 3; 100 μL/s).
2.4 Flow-cells

Flow-cell A was constructed by mounting a tight coil (28 mm diameter) of transparent PTFE tubing (0.8 mm i.d.; DKSH) onto a thin metal sheet (35 × 60 mm). The tubing at the centre of the coil passed through a slit in the sheet and was connected to a barbed plastic T-piece by slipping silicone tubing (1.02 mm i.d.; DKSH) over both the tubing and the fitting. The distance from the confluence point to the beginning of the coil was 1 cm (equivalent to approximately 5 μL).

A variety of flow-cell designs were explored by encasing different Teflon disks in a ‘GloCel’ chemiluminescence detector (Global FIA; Figure 11). The path of solution flow was set by channels (width 0.76 mm × depth 0.89 mm) machined into each disk. Solution inlet and outlet ports in the back plate of the detector were aligned with holes drilled though the disks. A sapphire window served as the transparent top surface of the flow-cell. After an appropriate back plate was fastened, an extended range photomultiplier module (Electron Tubes model P30A-05, ETP) was secured in the holding chamber with a nut and ferrule. When the disks contained a single solution inlet, the solutions were merged at a Y-piece outside the detector and the length between the confluence point and detection was approximately 4 cm.
(equivalent to approximately 20 μL). In cases where the disk contained two solution inlets, the confluence point was located directly in front of the photomultiplier tube.

![Figure 11. GloCel chemiluminescence detector (Global FIA) consisting of (i) flow-cell, (ii) PMT chamber, and (iii) nut and ferrule to secure the PMT and light-seal the chamber. Flow-cell components: (iv) flat gasket, (v) sapphire window, (vi) Teflon disk, (vii) 'O' ring and (viii) back plate (single-inlet design shown).]

Flow-cell B was a Teflon disk with machined single-inlet spiral channel (0.76 mm width × 0.89 mm depth, Figure 12i). The volume of the flow-cell was 275 μL.

Flow-cell C was a Teflon disk with machined single-inlet serpentine channel (0.76 mm width × 0.89 mm depth) containing 114 reversing turns (Figure 12ii). The volume of the flow-cell was 245 μL.

Flow-cell D was a modified version of flow-cell C, containing two solution inlets at the centre of the Teflon disk (Figure 12iii) and an appropriately adjusted back plate. The volume of the flow-cell was 235 μL.

Flow-cell E was constructed by replacing the Teflon disk with a spacer ring, forming a shallow open cylindrical space (Figure 12iv). The back plate was equipped with a custom designed Y-piece.
Flow-cell F was a modified version of flow-cell C, in which the rounded edges of the single-inlet serpentine channel were replaced with approximately right-angled turns.

Flow-cells G and H were identical to flow-cells C and D, except that the disks were constructed from Teflon impregnated with 25% glass microspheres to improve the transmission of light to the photomultiplier tube.

Flow-cell I, J and K were modified versions of flow-cell D containing mixing zones located shortly after (I: 1.0 mm diameter, J: 2.0 mm diameter) or at the confluence point (K: 4 mm diameter).

Flow-cell L was identical to flow-cell C, except that a 3 mm diameter well was removed from the centre of the Teflon disk and equipped with an appropriately adjusted back plate.

![Figure 12. Teflon disks with machined channels and inlet/outlet holes: flow-cells (i) B, (ii) C, (iii) D and (iv) E.](image)

With each change of flow-cell, the housing was re-sealed and the instrument was left for 40 minutes to avoid the temporary increase in baseline signal that was observed under the most sensitive settings.
3. Results and Discussion

3.1 Preliminary investigations into novel flow-cell configurations

The reaction of morphine with acidic potassium permanganate (in the presence of sodium polyphosphates) was selected for the comparison of different flow-cell configurations because it has been used extensively as a method of detection for flow analysis and HPLC [13, 25, 174, 175]. It is also a representative example of the relatively fast chemiluminescence reactions between strong oxidising agents and organic analytes [13]. The emission from this particular reaction occurs as a result of an electronically excited manganese(II) species and reaches maximum intensity within a few seconds under stopped flow conditions [174].

With the use of FIA methodology, morphine standards ($1 \times 10^{-8}$ M to $1 \times 10^{-5}$ M) were injected into a deionised water carrier stream, which merged with the permanganate reagent. The chemiluminescence intensities obtained using flow-cells B, C and D were similar to those obtained using the conventional coiled-tubing approach (flow-cell A). Flow-cell C gave the greatest signals, generally 6–11% superior to flow-cells A and B (Figure 13.). Flow-cell E was found to be significantly inferior in terms of chemiluminescence intensity (87% poorer than flow-cell A) and precision; the relative standard deviation (R.S.D) for 10 replicate injections of $1 \times 10^{-7}$ M morphine (9.6%) was much greater than that of the other configurations (all < 1.5%).
Figure 13. The effect of flow-cell configuration on chemiluminescence intensity (peak height) during the oxidation of morphine ($1 \times 10^{-7}$ M) with acidic potassium permanganate.

In contrast to the single-inlet cells (A, B, C and E, where mixing started at a T- or Y-piece located within close proximity to the inlet), flow-cell D allowed the reaction to be initiated directly in front of the photomultiplier tube. However, this design was unable to significantly improve the chemiluminescence response. This was further explored using the reactions between (i) tris(2,2'-bipyridine)ruthenium(III) and sodium hydroxide and (ii) luminol, hydrogen peroxide and potassium hexacyanoferrate(III), which both produce intense emissions of light suitable for visual observation (Figures 14 and 15). An examination of the chemiluminescence emission within flow-cell C revealed that some light may have been emitted prior to entry into the cell as demonstrated by the bright spot visible in the inlet hole (Figures 14i and 15i). Despite this, the most intense emission occurred within the first reversing turns of the serpentine design. In the case of flow-cell D, the two
solutions merged at the centrally located confluence point, delaying the onset of the chemiluminescence reaction and as a result, the maximum emission occurred several reversing turns away from this point (Figures 14ii and 15ii).

**Figure 14.** Photographic evidence of solution mixing efficiency in flow-cells (i) C and (ii) D, based on the distribution of light when solutions of tris(2,2'-bipyridine)ruthenium(III) and sodium hydroxide are continuously merged. An exposure time of 20 s was used for both photographs.

**Figure 15.** Photographic evidence of solution mixing efficiency in flow-cells (i) C and (ii) D, based on the distribution of light when solutions of luminol, hydrogen peroxide and potassium hexacyanoferrate(III) are continuously merged. An exposure time of 0.3 s was used for both photographs.

Flow-cells B and C were also compared to the conventional coiled-tubing approach (flow-cell A) using SIA methodology and the reaction of morphine (1×10^-7 M) with acidic potassium permanganate (5×10^-4 M). Deionised water (line 1,
2050 μL, 167 μL/s), morphine (line 2, 500 μL, 20 μL/s), and the permanganate reagent (line 3, 150 μL, 20 μL/s) were aspirated into the holding coil. The flow was then reversed to propel the reacting mixture (167 μL/s) through the detector. A comparison of the chemiluminescence signals obtained with flow-cells A, B and C (Figure 16, blue columns) revealed that the greatest signals were obtained from flow-cell A (15-35% superior to flow-cells B and C).

The manifold was then reconfigured (Figure 10) so that the solutions were aspirated directly through the detector, without reversing the flow of solution. This configuration is well-suited to chemiluminescence detection that involves relatively fast reactions, as mixing of solutions prior to entry into the flow-cell is minimised, enabling a greater proportion of the emitted light to be detected. The permanganate reagent (5 × 10^{-4} M; line 1, 150 μL, 167 μL/s) was aspirated prior to morphine (line 2, 1500 μL, 167 μL/s) and deionised water (line 3, 1000 μL, 100μL/s). Nine standard solutions (between 1 × 10^{-9} and 1 × 10^{-5} M) were tested using the three different flow-cells (A, B and C, Figure 16, pink columns). At equivalent concentrations of morphine, the signals obtained with flow-cell C were greater than those from flow-cells A and B by 7–23% and 75–104% respectively.
Figure 16. The effect of channel design on chemiluminescence signal (peak area) using traditional SIA (blue columns) and direct aspiration SIA methodologies (pink columns) during the oxidation of morphine ($1 \times 10^7$ M) with acidic potassium permanganate.

3.2 Further investigations into channel design

Several research groups have utilised chemiluminescence reaction zones consisting of meandering or spiral channels with right-angled turns (in flow-cells or microfluidic devices) [161, 162, 164, 171, 172]. A flow-cell comprising a spirally propagating serpentine channel with approximately 90° turns (hereafter referred to as flow-cell F), was compared with three previously described single-inlet flow-cell configurations, A, B (Figure 12i) and C (Figure 12ii).

Using FIA methodology, six morphine standards (between $1 \times 10^{-10}$ M and $1 \times 10^{-5}$ M) were injected into a deionised water carrier stream that merged with the permanganate reagent ($1 \times 10^{-3}$ M) at an external confluence point located within close proximity (4 cm, approximately 20 μL) to the inlet of the flow-cells. The
chemiluminescence intensities obtained using flow-cell C were again greater than the other configurations, although the difference across all of the cells was generally less than 10% (Figure 17, blue columns). Using SIA (direct aspiration of 150 µL of the permanganate reagent, 1500 µL of the morphine standard, and then 1000 µL of the deionised water carrier), the same trend was observed, but the differences in intensity were much greater (Figure 17, pink columns). Over five orders of magnitude ($1 \times 10^{-9}$ M to $1 \times 10^{-5}$ M), the signals obtained with flow-cell C were significantly greater than those from A (4-56%), B (51-76%) or F (11-103%).

![Figure 17](image)

*Figure 17.* The effect of channel design on chemiluminescence signals obtained with (i) FIA (peak height, blue columns) and (ii) direct aspiration SIA methodologies (peak area, pink columns) during the oxidation of morphine ($1 \times 10^{-7}$ M) with acidic potassium permanganate. R.S.D. was less than 1.4% for each flow-cell.

It appears that although the repeated approximately 90° turns of flow-cell F promote more efficient mixing than the steady curve of the spiral configuration (flow-cell B), the relatively sharp corners also create areas of poorer solution flow, resulting in inferior emission intensities compared to the serpentine design. A visual
examination of the chemiluminescence generated in flow-cell F revealed that air bubbles in the solution tended to remain in the corners of the channel, a problem also encountered in flow-through Z- or U-cells for photometric detection [176]. This problem became more apparent when using the relatively long-lived chemiluminescence reaction between luminol, hydrogen peroxide and potassium hexacyanoferrate(III), which produces nitrogen gas [173] (Figure 18).

![Photographic evidence of bubble formation within flow-cell F when solutions of luminol, hydrogen peroxide and potassium hexacyanoferrate(III) are continuously merged.](image)

**Figure 18.** Photographic evidence of bubble formation within flow-cell F when solutions of luminol, hydrogen peroxide and potassium hexacyanoferrate(III) are continuously merged.

### 3.3 Flow-cell material

Although chemiluminescence flow-cells and microfluidic reactors have often been constructed by machining channels into glass [165, 170, 171] or transparent polymer materials [163, 164, 166-168, 172], an investigation was undertaken to determine if the material used to construct these novel flow-cells had any impact on the proportion of light that can be captured by the photomultiplier tube. Using FIA methodology, a disk constructed from Teflon impregnated with 25% glass microspheres (flow-cell G) was compared to one of identical design constructed from standard Teflon (flow-cell C) and the conventional coiled-tubing (flow-cell A). The chemiluminescence reactions selected to examine these flow-cells were: (i) the
oxidation of morphine with acidic potassium permanganate [13] and (ii) the enhancement of luminol and hexacyanoferrate(III) by vanilmandelic acid [177]. The R.S.D for ten replicate injections of the $1 \times 10^{-7}$ M morphine standard solution was below 0.9% for all flow-cells. Across a wide range of analyte concentrations, 13-17% more light was captured using the Teflon disk impregnated with glass microspheres (Figure 19, blue columns). A similar trend was observed for the reaction between vanilmandelic acid and luminol, with signals from flow-cell G on average 14-27% superior to the other flow-cells (Figure 19, pink columns). This is attributed to the greater reflection of stray light towards the photodetector.

![Figure 19. The effect of the material used in flow-cell fabrication on chemiluminescence intensity (peak height) for the reactions between (i) morphine ($1 \times 10^{-7}$ M) and acidic potassium permanganate (blue columns) and (ii) vanilmandelic acid ($1 \times 10^{-3}$ M) and luminol (pink columns).](image)

3.4 Confluence point and mixing chamber

The position of the confluence point is crucial for chemiluminescence detection in FIA or HPLC methodologies that involve fast light-producing reactions. It is thought
that ideally, the solutions should merge as close as possible to the point of detection [53, 147, 178-180]. Despite the design of flow-cell D enabling solutions to be merged directly in front of the photodetector window, this design was unable to improve the transmission of light to the detector, even for the relatively fast oxidation of morphine with permanganate (Figure 13). This design was further explored by; (i) introducing mixing zones into the dual-inlet design, (ii) creating a larger mixing well in the centre of the detection zone by modifying both the disk and backing plate, and (iii) examining both the previously constructed and new flow-cell inserts with a wider range of rapid chemiluminescence reactions.

The modified dual-inlet disks had mixing zones of located shortly after the confluence point (1.0 mm diameter; flow-cell I, 2.0 mm diameter; flow-cell J), or at the confluence point (4.0 mm diameter; flow-cell K). The flow-cells were compared to the standard single-inlet (C) and dual-inlet (D) configurations using FIA methodology with the analytically useful reactions of: morphine with permanganate [25, 73]; codeine with tris(2,2'-bipyridine)ruthenium(III) [25, 181]; and arginine, ammonium chloride and urea with sodium hypobromite [168, 182-185]. The production of the emitting species in these reactions is rapid; with the maximum chemiluminescence intensity being obtained within a few microseconds to several seconds, under stopped flow conditions [41, 168, 174, 186]. For the reaction of morphine and permanganate, the greatest intensity was obtained with flow-cell C (Figure 20, blue columns). Interestingly, the chemiluminescence signal decreased as the size of the mixing zone was increased (a 13% to 16% reduction in light, compared to flow-cell C).

To overcome the poor temporal stability associated with using chemically oxidised tris(2,2'-bipyridine)ruthenium(III), the reagent was oxidised immediately prior to
each flow-cell test. Furthermore, to account for variations in signal intensity due to changes in the tris(2,2'-bipyridine)ruthenium(III) and hypobromite reagent concentrations over the day, each experiment was also simultaneously performed with a second FIA instrument (containing a coiled-tubing chemiluminescence detector) to obtain a relative response. A similar trend was observed across the six flow-cell configurations for the reaction between codeine and tris(2,2'-bipyridine)ruthenium(III). Flow-cell C again resulted in the greatest signal intensities and proved to be 10-19% superior to the modified dual-inlet flow-cells (I, J and K, Figure 20, pink columns).

![Graph showing the effect of mixing zones on chemiluminescence intensity](image)

**Figure 20.** The effect of mixing zones on chemiluminescence intensity (peak height) for the chemiluminescence reactions of (i) morphine with acidic potassium permanganate (blue columns) and (ii) codeine with tris(2,2'-bipyridine)ruthenium(III) (pink columns).

The reactions of ammonium, arginine and urea with hypobromite (Figure 21) provided responses analogous to those obtained with the permanganate and tris(2,2'-bipyridine)ruthenium(III) reaction systems. However, the
chemiluminescence intensities from flow-cells C and D were very similar and in the case of the reaction of ammonium with hypobromite, the greatest response was obtained with the dual-inlet configuration. This suggests that flow-cell D may prove to be advantageous for chemiluminescence reactions with extremely fast kinetics.

![Bar chart](image)

**Figure 21.** The effect of mixing zones on chemiluminescence intensity (peak height) for the reaction of ammonium chloride (blue columns), arginine (pink columns) and urea (yellow columns) with hypobromite.

A visual examination of the light emitted from flow-cells D, J and K using the reaction between tris(2,2'-bipyridine)ruthenium(III) and sodium hydroxide (Figure 22) revealed that the addition of mixing chambers delayed the onset of maximum emission (Figures 22v and vi). Furthermore, it is clear from the photographs that the solutions rapidly move through the path of least resistance within the mixing chambers, and only poor solution mixing occurred in surrounding areas.
Figure 22. Teflon disks (for GloCel detector) containing dual-inlet serpentine configuration channels with (i) no mixing zone (flow-cell D), (ii) 2.0 mm mixing zone after confluence point (flow-cell J), and (iii) 4.0 mm mixing zone at confluence point (flow-cell K). (iv-vi) Photographic evidence of solution mixing efficiency, based on the distribution of light when solutions of sodium hydroxide and tris(2,2'-bipyridine)ruthenium(III) are continuously merged. The exposure times for the three photographs were 0.8, 0.8 and 2.5 s, respectively.
To enhance solution mixing within a central reaction chamber, a 3 mm diameter well was removed from the centre of the serpentine flow-cell (flow-cell L) and two new back plates with integrated confluence points were created. The first contained angled inlets that met in a shallow well (4 mm diameter, 2.5 mm depth including both disk and back plate). The second contained tangential inlets located on opposing sides of the well with grooves to encourage vortex flow. This approach was similar to the mixing device employed by Kobayashi and Imai [187] to merge the reactant solutions of a peroxoalate chemiluminescence system prior to detection. However, under the stated conditions, these designs did not improve the chemiluminescence response (13% and 9% poorer than flow-cell C for the reaction of morphine with permanganate), presumably due to similar issues as those described above.
4. Conclusions

The exchange of disks in the GloCel detector provides a convenient way to optimise the flow-cell configuration for any particular chemiluminescence reaction. In general, the spirally propagating serpentine channel configuration (flow-cell C) was superior to the spiral (flow-cell B) and the square-serpentine designs (flow-cell F) in terms of generating the most intense emission. Even for relatively fast chemiluminescence reactions, the optimum position of the confluence point is often external to the detection zone. However, for some reactions, a dual-inlet configuration (flow-cell D) that enabled the solutions to be merged directly in front of the photodetector provided greater emission intensities. The introduction of larger chambers in the centre of the detection zone did not enhance the signal for any of the reactions investigated in this study. Disks constructed from Teflon impregnated with glass microspheres increased the quantity of light reaching the photodetector, highlighting the importance of flow-cell materials in minimising the loss of stray light.

The development of flow-cells that efficiently mix solutions and capture a greater proportion of the emitted light from rapid reactions will be particularly useful for chemiluminescence detection in HPLC. This will allow for the maximum emission to be obtained within a very small volume of solution, minimising the contribution of band broadening. The findings of this chapter have resulted in a recent publication [188].
Enhanced permanganate chemiluminescence

- Introduction
- Experimental
- Results and discussion
- Conclusion
CHAPTER THREE

1. Introduction

The emission of light from chemical reactions can be utilised for sensitive detection with relatively simple instrumentation in various flow-based analytical techniques [5, 13, 31, 43, 189-191]. However, its application is limited by the relatively small number of reactions with sufficient chemiluminescence quantum yields to be used under the conditions required for routine analysis. The intensity of the emission is determined not only by the concentration of the molecules and the overall quantum yield, but also by the reaction kinetics [68]. This aspect is crucial for chemiluminescence detection as analytes are merged with a reagent to initiate the chemiluminescence reaction, and only a small proportion of this solution is exposed to the photodetector at any particular moment [122, 192]. Ideally, the reaction should be sufficiently fast so that the majority of the emitted light can be captured before the analyte zone is flushed out of the detector.

One reagent that meets these requirements is acidic potassium permanganate, which over the last few decades has become widely used for detection in flow analysis based techniques [13]. The oxidation of various organic analytes with acidic potassium permanganate is known to be autocatalytic [193, 194] – the rate of reaction is influenced by the concentration of reaction intermediates or products - which has been attributed to the formation of manganese(II) and colloidal manganese(IV) [195-197].

Several researchers have described a change in permanganate chemiluminescence intensity or reaction rate in the presence of manganese(II) salts [15, 198-201]. Agater and co-workers added 200 mM manganese(II) to catalyse the slow oxidation of mono- and di-saccharides. The lowest analyte concentration that they examined was $1 \times 10^{-4}$ M with precipitation of manganese dioxide occurring at concentrations of
manganese(II) higher than 200 mM [198]. Zhu and co-workers determined manganese(II) based on its influence on the rate of the reaction between permanganate and 2,3-butanedione [199]. Townshend and co-workers added 1 mM manganese(II) to the carrier stream of their flow injection analysis system, which they found increased signal intensity by 16% for one analyte, but decreased the signal by 51% and 80% for two other analytes [15, 200, 201]. However, at that time, the emitting species in these reactions had not yet been confirmed [17, 202] (the luminescence was often erroneously attributed to the production of singlet oxygen [13, 15]) and the full implications of these observations were not apparent.

This chapter describes a systematic investigation into the relationship between the addition of reducing agents to freshly prepared acidic potassium permanganate solutions and the resulting impact on the intensity and rate of chemiluminescence reactions. These reagents were evaluated in terms of their stability and capacity to elicit analytically useful chemiluminescence in comparison with a conventional acidic potassium permanganate solution using a number of flow analysis techniques.
2. Experimental

2.1 Chemicals and reagents

Deionised water (Continental Water Systems, Victoria, Australia) and analytical grade reagents were used unless otherwise stated. Chemicals were obtained from the following sources: 3-aminophenol, amoxicillin, caffeic acid, dopamine hydrochloride, fenoterol hydrobromide, gallic acid, homovanillic acid, 4-nitrophenol, octopamine, quercetin, resveratrol, serotonin hydrochloride, sodium polyphosphate (+80 mesh), sodium thiosulfate, synephrine, tyramine, L-tyrosine and vanilmandelic acid from Sigma-Aldrich (New South Wales, Australia); ascorbic acid, manganese(II) sulfate and vanillin from BDH (Poole, England); potassium permanganate from Chem-Supply (South Australia, Australia); codeine, morphine, oripavine and pseudomorphine from GlaxoSmithKline (Victoria, Australia) and sulfuric acid from Merck (Victoria, Australia).

The ‘standard’ permanganate reagent was prepared by dissolution of potassium permanganate ($1 \times 10^{-3}$ M) in 1% (m/v) sodium polyphosphate and adjusting to pH 2.5 with sulfuric acid.

The ‘enhanced’ permanganate reagent was prepared by dissolution of potassium permanganate ($1.0 \times 10^{-3}$ M) in 1% (m/v) sodium polyphosphate and adjusting to pH 2.5 with sulfuric acid and then adding either solid manganese(II) sulfate or sodium thiosulfate, using a small volume of a 0.1 M solution.

Stock solutions ($1 \times 10^{-3}$ M) of the analytes were prepared and diluted in deionised water and sonicated to aid dissolution. Opiate alkaloid stocks ($1 \times 10^{-3}$ M) were prepared in acidified deionised water.
2.2 Flow injection analysis

The FIA manifold was constructed from a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Victoria, Australia) with bridged PVC pump tubing (1.02 mm i.d.; DKSH, Queensland, Australia), black manifold tubing (0.76 mm i.d., Global FIA, Washington, USA), six-port injection valve (Vici 04 W-0192L, Valco Instruments, Houston, Texas, USA) and GloCel chemiluminescence detector (Global FIA, Washington, USA) equipped with either flow-cell C (single-inlet serpentine) or D (dual-inlet serpentine) and mounted flush against the window of an extended range photomultiplier module (Electron Tubes P30A-05, ETP, New South Wales, Australia). All tubing entering and exiting the detector was black PTFE (0.76 mm i.d., Global FIA). The output signal from the detector was recorded with an ‘e-corder 410’ data acquisition system (eDAQ, New South Wales, Australia).

The analyte standards were injected (70 μL) into a deionised water carrier stream that merged with the chemiluminescence reagent, shortly before or within the flow-cell (Figure 23). Solution flow rates were optimised over the range 0.9 mL/min to 3.5 mL/min per line.

![Figure 23. Schematic of the FIA manifold.](image-url)
2.3 Flow-cells

The single-inlet and dual-inlet serpentine flow-cells (C and D, Figures 12ii and 12iii) were utilised in this study. They were encased in a GloCel chemiluminescence detector (as described in Chapter 2, section 2.4).

2.4 Stopped flow

Stopped flow analysis experiments were performed with an FIA manifold constructed from a programmable dual-syringe pump (Model sp210iw, World Precision Instruments, Victoria, Australia), Valco six-port injection valve, and GloCel chemiluminescence detector (Global FIA) equipped with either flow-cell C or flow-cell D (Figure 24). The carrier and reagent syringes (Terumo, 10 mL Luer lock) were loaded with deionised water and the permanganate reagent. After the injection loop was filled with the analyte solution, the pump was activated. Equivalent, precise volumes of the carrier and reagent solutions were dispensed, which propelled the analyte and reagent into the serpentine reaction channel, where it was held for a set period of time to obtain the entire chemiluminescence intensity versus time profile. The output signal from the photomultiplier module was recorded with an ‘e-corder 410’ data acquisition system (eDAQ, NSW, Australia).

Longitudinal dispersion of the analyte zone was minimised using the shortest possible length of tubing between the valve and detector. By inserting a small volume of analyte solution into a carrier stream, rather than dispensing an analyte stream from the syringe pump, convenient and thorough flushing of the detector was achieved. Activating the pump for an extended period of time without filling the injection loop after each profile was collected, ensured that the manganese(II) product of the reaction did not affect subsequent tests.
2.5 Sequential injection analysis

Analyte standards were reacted with the permanganate reagent by sequential aspiration of solutions through a multi-position valve (model C25Z, Valco) and the GloCel detector equipped with flow-cell C, using a milliGAT pump (Global FIA, Figure 25). A desktop computer equipped with data acquisition board (LabJack U12, National Instruments, Victoria, Australia) and LabView Software (Version 8.0, National Instruments) was used to control the pump, valve, and record the output from the photomultiplier module. This automated system was programmed to repeatedly aspirate 150 μL of reagent (valve position 1; 167 μL/s), 1500 μL of the analyte standard (position 2; 167 μL/s), and 1000 μL of deionised water (position 3; 100 μL/s), with a 60 min pause after every fifth cycle.

Figure 25. Schematic of the SIA manifold.
2.6 UV-Visible spectrometry

UV-Visible absorbance spectra were collected using a Cary 300 Bio UV-visible spectrophotometer (Varian, Victoria, Australia) with 10 mm path length, sealable quartz cuvettes (Starna, New South Wales, Australia). A solution of acidic potassium permanganate \((1 \times 10^{-3} \text{ M})\) was prepared, and immediately following the addition of sodium thiosulfate (0.6 mM), the reagent was transferred into a cuvette within the spectrophotometer, where absorption spectra (wavelengths between 300-700 nm) were taken once every hour for 48 hours.
3. Results and Discussion

3.1 Manganese(II)

3.1.1 Preliminary chemiluminescence investigations

Acidic potassium permanganate solutions containing sodium polyphosphate have limited stability due to both the degradation of the oxidant [203] and a pH dependent hydrolysis of the polyphosphate [204], a process which is accelerated by exposure to natural light. The stability of a ‘standard’ permanganate reagent (1.0 mM, 1% m/v polyphosphate, pH 2.5) was assessed using automated SIA methodology. The reagent, analyte (morphine, $1 \times 10^{-6}$ M) and deionised water carrier were sequentially aspirated through flow-cell C, with a 60 minute pause after every fifth cycle. This approach minimises the mixing of solutions prior to entry into the flow-cell, enabling detection of a greater proportion of the emitted light. Over a 48 hour period, a 26% increase in chemiluminescence intensity was observed (R.S.D. of the 49 sets of 5 replicates was 5.1%).

![Chemiluminescence signal over time](image)

**Figure 26.** Stability of the standard permanganate reagent (1.0 mM) with morphine ($1 \times 10^{-6}$ M), over 48 hours with SIA methodology.
Slezak *et al.* [205] recently demonstrated that acidic potassium permanganate solutions exposed to natural light for up to six months can improve the chemiluminescence emissions for several simple phenols. In this case, significant degradation of both the oxidant and enhancer (polyphosphates) was counteracted by the manganese(II) catalysis of the light-producing pathway resulting in dramatic increases in the reaction kinetics for these organic analytes [205].

The addition of manganese(II) sulfate to the standard reagent results in the partial reduction of permanganate to form a concomitant mixture of both manganese(VII) and manganese(III) [205]. This effectively replicates the reduction process outlined by Slezak, without the lengthy time frame. The presence of polyphosphates in the reagent is crucial in preventing the formation and subsequent flocculation of a murky red manganese dioxide suspension which is characteristic of the Guyard reaction [206].

The viability of this method of reagent preparation was assessed *via* repeated reaction of morphine \((1 \times 10^{-6}\text{ M})\) with a reagent containing 1.0 mM permanganate and 0.6 mM manganese(II) sulfate over 72 hours using SIA methodology. An immediate 3-fold increase in chemiluminescence intensity was observed after the first hour, which became even greater (8.3-fold) 24 hours after reagent preparation (Figure 27). During the ensuing 48 hours, minimal variation in signal intensity was observed (R.S.D. was 1.1%), and therefore all subsequent experiments were performed using reagents left to equilibrate for 24 hours. The significant increase in chemiluminescence intensity resulted in this reagent being denoted ‘enhanced’ permanganate.

Townshend and co-workers [15, 200, 201] have previously reported relatively small levels of enhancement (or even inhibition) of the chemiluminescence response
for several analytes when manganese(II) was used in flowing systems. However, this may have been attributable to merging solutions of manganese(II), permanganate (without polyphosphates) and the analyte online (which allowed very little time for the equilibration of manganese species).

![Chemiluminescence signal (arbitrary units) vs Time (h)](image)

**Figure 27.** Stability of the enhanced permanganate reagent (1.0 mM containing 0.6 mM manganese(II) sulfate) with morphine ($1 \times 10^{-6}$ M), over 48 hours with SIA methodology.

### 3.1.2 Optimisation of reagent conditions

An evaluation of reagent conditions was then conducted using FIA methodology to determine the optimum concentration of manganese(II) required to produce the greatest enhancement in chemiluminescence response with two model analytes, morphine and the adrenergic amine synephrine.

A set of six reagents were prepared, containing 1.0 mM permanganate, 1% (m/v) sodium polyphosphate, adjusted to pH 2.5 using sulfuric acid. Concentrations of manganese(II) ranged from 0.0 mM to 1.0 mM in each of the reagents. The optimal concentration of manganese(II) appeared to be analyte dependent (Figure 28). A
moderate increase in chemiluminescence intensity was obtained for morphine (7-fold), whilst a significant 80-fold increase was observed for synephrine.

**Figure 28.** The effect of manganese(II) sulfate concentration on the chemiluminescence response (peak height) obtained from the reaction of acidic potassium permanganate (1.0 mM) with (i) morphine ($1 \times 10^{-5}$ M) and (ii) synephrine ($1 \times 10^{-5}$ M) using FIA.
The greater chemiluminescence intensities obtained for both morphine and
sympathomimetic with the enhanced reagent also translated into much lower limits of
detection for both analytes, $4.6 \times 10^{-11}$ M and $1.2 \times 10^{-9}$ M, respectively. This
represents over an order of magnitude greater sensitivity than that obtained using the
standard reagent (Tables 1 and 2) and is superior to all previously reported detection
limits obtained with acidic potassium permanganate for these analytes [13, 21, 22].

Table 1. Analytical figures of merit obtained for morphine with the standard and
enhanced permanganate reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibration function (peak height, mV)</th>
<th>$R^2$</th>
<th>LOD (M)$^a$</th>
<th>R.S.D. %$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard permanganate</td>
<td>$y = 0.928x + 8.588$</td>
<td>0.9962</td>
<td>$5.4 \times 10^{-6}$</td>
<td>1.3</td>
</tr>
<tr>
<td>Enhanced permanganate</td>
<td>$y = 0.847x + 8.512$</td>
<td>0.9985</td>
<td>$4.6 \times 10^{-11}$</td>
<td>0.69</td>
</tr>
</tbody>
</table>

$^a$Calculated as $3 \times$ the standard deviation of the blank response.

$^1 \times 10^{-7}$ M (n = 10)

Table 2. Analytical figures of merit obtained for sympathomimetic with the standard and
enhanced permanganate reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibration function (peak height, mV)</th>
<th>$R^2$</th>
<th>LOD (M)$^a$</th>
<th>R.S.D. %$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard permanganate</td>
<td>$y = 0.962x + 7.538$</td>
<td>0.9989</td>
<td>$1.6 \times 10^{-8}$</td>
<td>0.89</td>
</tr>
<tr>
<td>Enhanced permanganate</td>
<td>$y = 0.721x + 6.397$</td>
<td>0.9993</td>
<td>$1.2 \times 10^{-9}$</td>
<td>0.58</td>
</tr>
</tbody>
</table>

$^a$Calculated as $3 \times$ the standard deviation of the blank response.

$^1 \times 10^{-6}$ M (n = 10)

3.1.3 Analyte screening test

Sixteen compounds from four important classes of analytes (antioxidants [19, 20],
neurotransmitters [24, 207], adrenergic amines [21, 22] and opiate alkaloids [27, 153]) that have been previously detected with acidic potassium permanganate were
selected to examine the influence of the enhanced reagent on chemiluminescence signals (Figure 29).

![Chemical structures](image)

**Figure 29.** Structures of the sixteen compounds (antioxidants, neurotransmitters, adrenergic amines and opiate alkaloids) used in the reagent screening test.

Moderate increases in chemiluminescence intensity (less than an order of magnitude) were obtained for phenols containing ortho-hydroxy or -alkoxy groups
(such as morphine, dopamine, resveratrol, quercetin and caffeic acid) when using the enhanced reagent (Figure 30 and Table 3). Significant enhancement (between 20- and 95-fold) was obtained for the simple phenol hordenine and three closely related compounds (octopamine, synephrine and tyramine). The enhanced reagent affords a significant improvement in sensitivity, which may allow for the detection of compounds not traditionally examined with acidic potassium permanganate chemiluminescence.

![Chemiluminescence intensities](image)

**Figure 30.** Chemiluminescence intensities (peak heights) for selected analytes with standard permanganate (1.0 mM; blue columns) and enhanced permanganate (1.0 mM containing 0.6 mM manganese(II) sulfate; pink columns) using HIA.

### 3.1.4 Stopped flow analysis

Stopped flow analysis was used to obtain the chemiluminescence intensity versus time profile for synephrine (1 × 10^{-5} M) using the standard reagent (Figure 31).
Despite acidic potassium permanganate having previously been applied for the post-column determination of synephrine [21, 22], the kinetics of this reaction are not ideal for flow-through detection (Figure 31i). The maximum emission intensity occurred between 10 and 35 seconds, and did not decay to half its maximum until 40-80 seconds. However, using an enhanced reagent containing 0.1 mM manganese(II), the time taken to reach maximum intensity decreased to 2.4 seconds (Figure 31ii). Increasing the manganese(II) concentration between 0.3 mM and 1.0 mM (Figure 31iii and Figure 31iv) resulted in small additional increases in reaction rate, which were accompanied by improvements in maximum intensity (peak height), but decreases in the total chemiluminescence emission (peak area) (Figure 32).

**Figure 31.** Chemiluminescence intensity *versus* time profiles for the reaction of synephrine \((1 \times 10^{-2}) \text{ M}\) with acidic potassium permanganate containing (i) 0.0 mM, (ii) 0.1 mM, (iii) 0.3 mM, and (iv) 0.6 mM manganese(II) sulfate.
Figure 32. Effect of manganese(II) sulfate concentration on (i) maximum chemiluminescence intensity (peak height), and (ii) total emission (peak area) for the reaction of acidic potassium permanganate with synephrine ($1 \times 10^{-5}$ M).

The distribution of light within the single inlet serpentine reaction channel (flow-cell C) was examined by continuously merging the reactant solutions at the external confluence point and capturing the emitted light with a digital SLR camera (Figure 33). The chemiluminescence emission from the reaction between the standard reagent and synephrine ($1 \times 10^{-3}$ M) was spread throughout the flow-cell, with a slightly more intense emission emanating from the outer coil of the serpentine channel (Figure 33i). In agreement with the intensity versus time profile (Figure 31i), it appears that the reaction mixture would continue to emit light for a significant period of time after exiting the detection zone. In contrast, use of the enhanced reagent resulted in an intense emission in the centre of the detection zone (Figures 33ii and 33iii), which decreased considerably before the solution exited the cell.
Although the reaction between morphine and the standard permanganate reagent is already rapid (Figure 33iv), an increase in reaction rate was observed upon reaction with the enhanced reagent, producing more intense chemiluminescence in the inner coil of the serpentine channel (Figures 33v and 33vi).

![Chemiluminescence emissions](image)

**Figure 33.** Chemiluminescence emissions occurring within flow-cell C of the GloCel detector. Photographs (i), (ii), and (iii) show the reaction between synephrine ($1 \times 10^{-3}$ M) and acidic potassium permanganate, with 0.0 mM, 0.3 mM, and 0.6 mM manganese(II) sulfate, respectively, using an exposure time of 5 min. Photographs (iv), (v), and (vi) show the reaction of morphine ($1 \times 10^{-3}$ M) under the same three reagent conditions and an exposure time of 2.5 s.

### 3.1.5 Flow-cell comparisons

As discussed in chapter 2, the design of chemiluminescence detectors is crucial in obtaining optimal mixing efficiency to maximise the chemiluminescence response [73, 188]. Despite morphine being an exemplar of the many fast chemiluminescence reactions between oxidising agents and organic analytes [13], a flow-cell designed to allow the reaction to be initiated in front of the photomultiplier tube (flow-cell D) failed to improve the chemiluminescence response. However, the reaction of hypobromite and ammonium chloride has extremely rapid reaction kinetics and in
this instance, flow-cell D proved superior (by 3%) to an approach utilising an external confluence point (flow-cell C). The results obtained for synephrine using stopped flow analysis indicated that considerable increases in reaction rate and chemiluminescence intensity were obtained using the enhanced reagent. To examine the influence of increased reaction rates on the position of maximum chemiluminescence emission, the aforementioned flow-cells (C and D) were compared to the conventional coiled-tubing approach (flow-cell A) using FIA methodology.

Unlike the standard permanganate reagent (Figure 34, blue columns), the reaction of morphine with the enhanced reagent produced greater signals with flow-cell D (reaction initiated in front of the detector) than the other configurations (A or C), by 27% and 14% respectively (Figure 34, pink columns).

**Figure 34.** Comparison of chemiluminescence flow-cells for the reaction of morphine ($1 \times 10^{-3}$ M) with (i) standard permanganate (blue columns) and (ii) enhanced permanganate (pink columns) using FIA. The R.S.D. ($n = 10$) for each configuration was less than 1.8%.
Under stopped flow conditions, the chemiluminescence intensity *versus* time profile for the reaction of morphine \((1 \times 10^{-3} \text{ M})\) with the standard reagent reached a maximum after 2 seconds with both flow-cells C and D. The overall signal (peak area) obtained using flow-cell C with an external Y-piece was 5% greater than that of flow-cell D (Figure 35i, blue trace). The enhanced reagent halved the time taken to reach maximum emission. Using flow-cell D, an 18% increase in area and a 260% increase in height was obtained (Figure 35ii, pink trace). This is attributable to the dual-inlet design allowing initiation of reaction in front of the photomultiplier tube and the increased reaction kinetics. The increases were smaller for flow-cell C: 1.3% and 180%, respectively, indicating that in this configuration, a significant proportion of the emission (approximately 15%) occurred before the reacting mixture entered the detection zone (Figure 35ii, blue trace). It was concluded that flow-cell D was the most suitable configuration for reactions with such rapid reaction kinetics and it was therefore used for all future experiments with the enhanced reagent.

*Figure 35.* Chemiluminescence intensity *versus* time profiles for the reaction between \(1 \times 10^{-3} \text{ M}\) morphine and acidic potassium permanganate with flow-cells (i) C (blue) and D (pink dotted) and (ii) C and D after addition of 0.6 mM manganese(II) sulfate.
3.2 Sodium thiosulfate

3.2.1 Preliminary experiments

In 1989, Perez-Benito et al. [208] demonstrated that the addition of sodium thiosulfate to a solution of potassium permanganate (without polyphosphates) produced a transparent brown solution of colloidal manganese(IV). This reagent has been used as an oxidant in reactions with formic acid [209], oxalic acid [210], lactic acid [211] and l-tryptophan [212]. Recently, Hindson et al. [213] discovered that when 1% (m/v) polyphosphates are added to the acidic potassium permanganate reagent solution prior to the addition of aqueous sodium thiosulfate, the result is a near instantaneous partial reduction of permanganate to form a concomitant mixture of both manganese(VII) and manganese(III).

An optimisation of the permanganate and thiosulfate concentrations required to produce the greatest chemiluminescence intensities with morphine and synephrine was undertaken using FIA. A series of twenty reagents were prepared containing concentrations of potassium permanganate between 1.0 and 1.9 mM, thiosulfate between 0.0 and 1.5 mM and 1% (m/v) sodium polyphosphates. The partial reduction of permanganate was accompanied by a visual colour change; as the concentration of thiosulfate added to the reagent increased, the characteristic purple colour diminished.

Whilst the optimal concentrations of oxidant and reductant appeared to be analyte dependant (Figures 36 and 37), in general greater concentrations of permanganate required the addition of more thiosulfate to achieve similar levels of enhancement. However, concentrations of thiosulfate greater than 1.0 mM generally resulted in
significant decreases in chemiluminescence intensity due to the complete reduction of permanganate.

Moderate increases (5- to 9-fold) in signal intensity were achieved following the addition of 0.3 mM thiosulfate (to the four permanganate reagents) and subsequent reaction with a morphine standard (1 × 10^{-7} M). Further enhancement (between 11- and 18-fold) was obtained when the concentration of thiosulfate was increased to 0.6 mM. Interestingly, the addition of 1.0 mM thiosulfate resulted in quenching of the chemiluminescence signal, except for the reagent containing 1.9 mM permanganate, where a 17-fold increase in intensity was observed (Figure 36).

![Figure 36](image)

*Figure 36.* The effect of thiosulfate concentration on the chemiluminescence response (peak height) obtained for the reaction of four acidic potassium permanganate reagents (i) 1.0 mM, (ii) 1.3 mM, (iii) 1.6 mM and (iv) 1.9 mM with morphine (1 × 10^{-5} M), using FIA methodology.

Superior levels of enhancement were achieved when the twenty permanganate reagents were reacted with synephrine (1 × 10^{-5} M, Figure 37). As with morphine, moderate increases in signal intensity were achieved with the reagents containing
0.3 mM thiosulfate (37- to 45-fold). Significant enhancement was achieved with reagents containing 0.6 mM thiosulfate (63-fold from a 1.9 mM permanganate solution, Figure 37iv).

![Graph showing the effect of thiosulfate concentration on chemiluminescence intensity](image)

**Figure 37.** The effect of thiosulfate concentration on the chemiluminescence response (peak height) obtained for the reaction of four acidic potassium permanganate reagents (i) 1.0 mM, (ii) 1.3 mM, (iii) 1.6 mM and (iv) 1.9 mM with synephrine (1 × 10⁻⁵ M), using FIA methodology.

3.2.2 *Stopped flow analysis*

Stopped flow experiments were conducted to examine the change in reaction kinetics with increasing thiosulfate concentration. Five permanganate reagents (1.9 mM) were prepared with concentrations of thiosulfate between 0.0 and 1.5 mM and were subsequently reacted with a synephrine standard (1 × 10⁻⁵ M).

As with the previous mode of preparation (utilising the addition of manganese(II)), the production of the enhanced reagent using thiosulfate significantly increased the rate of the reaction between synephrine and permanganate. This was reflected in the
chemiluminescence versus time profiles (Figure 38), with a significant reduction in the time required to reach maximum emission (from 60 seconds to 3 seconds) and increases in both peak intensity (peak height) and overall light emitted (peak area, Table 3). Concentrations of thiosulfate greater than 1.0 mM resulted in notable decreases in both peak intensity and overall signal, until quenching of the signal was obtained (1.5 mM thiosulfate) due to the complete consumption of permanganate. The enhancement of chemiluminescence signals for compounds such as synephrine (that react slowly with the standard reagent) was of greatest interest to this study and therefore all subsequent experiments were conducted using an enhanced reagent containing 1.9 mM permanganate and 0.6 mM thiosulfate.

Figure 38. Chemiluminescence intensity versus time profiles for the reaction of synephrine (1 × 10⁻⁵ M) with acidic potassium permanganate (1.9 × 10⁻⁵ M) containing (i) 0 mM, (ii) 0.3 mM, (iii) 0.6 mM, and (iv) 1.0 mM sodium thiosulfate.
Table 3. Effect of sodium thiosulfate concentration on the chemiluminescence intensity (peak height) and total chemiluminescence emission (peak area) from the reaction of acidic potassium permanganate ($1.9 \times 10^{-3}$ M) with syneprine ($1 \times 10^{-5}$ M).

<table>
<thead>
<tr>
<th>Thiosulfate concentration (mM)</th>
<th>Chemiluminescence intensity (mV)</th>
<th>Total chemiluminescence emission (mV.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>10</td>
<td>838</td>
</tr>
<tr>
<td>0.3</td>
<td>468</td>
<td>1686</td>
</tr>
<tr>
<td>0.6</td>
<td>498</td>
<td>1558</td>
</tr>
<tr>
<td>1.0</td>
<td>179</td>
<td>605</td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3.3 Stability studies

The stability of the enhanced reagent was assessed by monitoring the chemiluminescence intensity from the reaction with a morphine standard ($1 \times 10^{-6}$ M) using SIA. A set of five replicate injections was performed each hour for 48 hours following the addition of thiosulfate to the regent solution (Figure 39). Unlike the previous mode of reagent preparation (utilising the addition of manganese(II)), the addition of thiosulfate produced an immediate increase in the chemiluminescence signal (11-fold) that did not change significantly over 48 hours (a change of < 1% based on the line of best fit). The R.S.D. for the 49 sets of five signals was 0.8%. Similar results were obtained when monitoring the reaction with syneprine for 18 hours (< 0.1% change; 1.3% R.S.D.).
Figure 39. Stability of the enhanced permanganate reagent (1.9 mM containing 0.6 mM thiosulfate) with morphine (1 x 10^{-6} M), over 48 hours with SIA methodology. The first point (t = 0) was obtained using the permanganate reagent prior to the addition of thiosulfate.

The stability of the enhanced reagent was also assessed by monitoring its visible absorbance over 48 hours (Figure 40). A minor decrease in the structured band at approximately 525 nm (characteristic of permanganate) and no fluctuation at lower wavelengths, (where a large absorbance is typical of manganese(III) complexes) confirmed that the enhanced reagent is extremely stable over an extended period of time.
Figure 40. Absorption spectra for the enhanced permanganate reagent (1.9 mM, containing 0.6 mM thiosulfate) measured every hour for 48 hours (only every sixth spectrum shown). The initial spectrum is shown in bold.

3.2.4 Reagent comparison and screening test

The previously observed improvement in chemiluminescence intensity obtained using the manganese(II) enhanced reagent demonstrated that the greatest levels of enhancement are achieved with simple phenols (such as synephrine), rather than those containing ortho-hydroxy or -alkoxy groups (such as morphine). A comparison was undertaken to examine the improvements in chemiluminescence intensities obtained from the permanganate reagents (standard, manganese(II) enhanced, and thiosulfate enhanced) upon reaction with several simple phenols (Figure 41) using FIA methodology.
Significant increases in chemiluminescence intensity were obtained for both of the enhanced reagents, however, the thiosulfate method proved on average 16% superior to the manganese(II) preparation. This analyte screen confirmed that; (i) thiosulfate is a viable alternative to manganese(II) as an enhancer for permanganate chemiluminescence, and (ii) increases of well over an order of magnitude can be obtained for a wide range of phenolic compounds (Figure 42). The greatest improvements in chemiluminescence intensity were obtained for the phenolic amino acid tyrosine (164-fold) and the sympathomimetic β-adrenergic agonist, fenoterol (118-fold, Table 4).
Figure 42. Chemiluminescence intensities (peak heights) for selected analytes with the standard reagents (1.0 mM potassium permanganate, blue columns and 1.9 mM potassium permanganate, pink columns) and the enhanced reagents (1.0 mM potassium permanganate containing 0.6 mM manganese(ll), yellow columns and 1.9 mM potassium permanganate containing 0.6 mM thiosulfate, green columns) using FIA. All analytes were tested at a concentration of $1 \times 10^{-5}$ M, except for morphine ($1 \times 10^{-6}$ M).

Table 4. Chemiluminescence intensities (peak heights) for reactions between various analytes ($1 \times 10^{-5}$ M) and the standard reagent (1.0 mM permanganate) and the enhanced reagent (containing 0.6 mM thiosulfate) using FIA.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Chemiluminescence intensity (mV)</th>
<th>Enhancement factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard permanganate</td>
<td>Enhanced permanganate</td>
</tr>
<tr>
<td>Vanillin</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>3</td>
<td>177</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
<td>164</td>
</tr>
<tr>
<td>Tyramine</td>
<td>11</td>
<td>485</td>
</tr>
<tr>
<td>3-Aminophenol</td>
<td>10</td>
<td>146</td>
</tr>
<tr>
<td>Synephrine</td>
<td>14</td>
<td>679</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>3</td>
<td>355</td>
</tr>
<tr>
<td>Morphine a</td>
<td>84</td>
<td>698</td>
</tr>
</tbody>
</table>
3.2.5 Detection limits

The greater chemiluminescence intensities obtained for both fenoterol and tyrosine with the enhanced reagent also translated into much lower limits of detection for both analytes, $1 \times 10^{-9}$ M and $4 \times 10^{-9}$ M, respectively. This represents a significant increase in sensitivity compared to that obtained with the standard reagent (Tables 5 and 6). Furthermore, the detection limit for tyrosine is superior to all previously reported values obtained with acidic potassium permanganate [23]. Fenoterol has not previously been detected with permanganate chemiluminescence.

| Table 5. Analytical figures of merit obtained for fenoterol with the standard and enhanced permanganate reagents. |
|----------------|----------------|--------|-----------------|--------|
| Reagent        | Calibration function (peak height, mV) | $R^2$  | LOD (M)$^a$      | R.S.D. %$^a$ |
| Standard permanganate | $y = 0.862x + 4.715$ | 0.9969 | $4.8 \times 10^{-8}$ | 0.95  |
| Enhanced permanganate | $y = 0.839x + 6.542$ | 0.9985 | $1 \times 10^{-9}$ | 0.56  |

$^a$Calculated as $3 \times$ the standard deviation of the blank response.

$^1 \times 10^{-5}$ M (n = 10)

| Table 6. Analytical figures of merit obtained for tyrosine with the standard and enhanced permanganate reagents. |
|----------------|----------------|--------|-----------------|--------|
| Reagent        | Calibration function (peak height, mV) | $R^2$  | LOD (M)$^a$      | R.S.D. %$^a$ |
| Standard permanganate | $y = 0.908x + 4.429$ | 0.9916 | $2.3 \times 10^{-7}$ | 1.05  |
| Enhanced permanganate | $y = 0.835x + 6.154$ | 0.9983 | $3.6 \times 10^{-9}$ | 0.61  |

$^a$Calculated as $3 \times$ the standard deviation of the blank response.

$^1 \times 10^{-5}$ M (n = 10)
4. Conclusions

Acidic potassium permanganate chemiluminescence has been used to detect a wide variety of compounds, including biomolecules, antioxidants, neurotransmitters, opiate alkaloids and pharmaceuticals. The greater control of reaction rates and enhanced emission intensities obtained using manganese(II) or sodium thiosulfate catalysis will improve the detection compatibility of various flow analysis methodologies. This approach not only provides the potential for increased sensitivity in existing applications of acidic potassium permanganate chemiluminescence detection, but may also enable its extension to a multitude of new analytes and analytical techniques. The findings of this chapter have resulted in two recent publications [205, 213].
Direct detection of biologically significant thiols and disulfides with post-column chemiluminescence

- Introduction
- Experimental
- Results and discussion
- Conclusion
1. Introduction

Over the past two decades there has been a growing interest in the quantification of low-molecular mass thiols and disulfides as researchers gain new insight into their role in significant cellular processes [85, 88, 97-99, 108, 112, 113, 116, 117, 121, 214, 215]. Particular attention has been paid to the redox couple, glutathione (GSH) and glutathione disulfide (GSSG). Oxidation of the free thiol to the corresponding disulfide is amongst the earliest responses to increases in reactive oxygen species in eukaryotic cells, and therefore assessment of the GSH/GSSG ratio offers great potential for diagnosing and monitoring pathological and physiological conditions related to oxidative stress [85, 97, 99, 112, 113].

From the numerous methods previously developed for the detection of thiols and disulfides, several key analytical challenges have emerged [84, 85, 87, 107, 109, 110, 114, 115]. The analytes typically lack strong chromophores or fluorophores, which is most commonly overcome by derivatisation of the free thiols before chromatographic/electrophoretic analysis, and then repeating the procedure on a second sample aliquot after reduction of the disulfides to their respective thiols [84, 85, 87, 107, 109, 110, 114, 115]. The disulfide concentrations are then calculated from the difference between the measurements obtained in the two separate analytical steps. This approach is not only time consuming, but also vulnerable to several major sources of error [87, 115], the most important of which is the oxidation of thiols during either the initial sample deproteinisation, or under the alkaline conditions often employed for derivatisation and reduction steps. Disulfides are present in biological samples at much lower concentrations than thiols, and even a small degree of auto-oxidation during these extensive sample preparation procedures can lead to their considerable overestimation [84, 85, 87, 106-109, 116, 117].
Therefore, the determination of biologically significant thiols and disulfides should ideally combine minimal sample handling (under conditions that prevent auto-oxidation) with direct detection of all target analytes in a single chromatographic separation. Thiols and disulfides have previously been detected without derivatisation using electrochemistry, mass spectrometry and fluorescence quenching [84, 87, 107, 110, 116-119]. Whilst these modes of detection offer advantages over the more popular derivatisation techniques in terms of procedural simplicity, their application is limited by equipment complexity, cost, electrode stability and/or analysis time [108, 110, 120, 121].

Several procedures coupling a separation with chemiluminescence detection have been developed for the direct determination of thiols [18, 123, 124]. The post-column chemiluminescence oxidation of GSH and cysteine with cerium(IV) (sensitised by rhodamine B) was reported by Li et al. [123], however this methodology resulted in unsatisfactory resolution of the two analyte peaks. Zhao et al. [124] described the use of microchip electrophoresis and luminol chemiluminescence to determine GSH, cysteine and haemoglobin in red blood cells. McDermott et al. [18] reported a procedure to determine GSH in cultured muscle cells, based on HPLC with direct acidic potassium permanganate chemiluminescence detection. However, like other previously reported chemiluminescence-based detection systems for thiols [123, 124], the reagent was not suitable to detect disulfides in biological systems, and therefore the quantification of GSGG required thiol blocking and disulfide bond reduction steps prior to a second chromatographic run [18].

In recent years, there have been several major advances in two of the most prolifically used chemiluminescence reagent systems (acidic potassium permanganate and tris(2,2’-bipyridine)ruthenium(III)), in terms of their stability [33,
205, 213] and sensitivity [205, 213], and the understanding of their light producing pathways [14, 216, 217]. Furthermore, the development of a colloidal manganese(IV) chemiluminescence reagent [46, 48] which has been found to produce light with a wider range of compounds than other manganese based oxidants offers the potential for new detection strategies of native (underivatised) analytes.

This chapter presents a systematic evaluation of the relative chemiluminescence responses from several biologically significant thiols and disulfides (Figure 43) with conventional and new adaptations of both the permanganate and tris(2,2′-bipyridine)ruthenium(III) reagents, in addition to the manganese(IV) colloid. A chromatographic separation of these analytes was subsequently developed, employing chemiluminescence detection to assess the redox state of whole blood from twelve healthy volunteers.

![Chemical Structures](image)

**Figure 43.** Structures of biologically significant thiols and disulfides.
CHAPTER FOUR

2. Experimental

2.1 Chemicals and reagents

Deionised water (Continental Water Systems, Victoria, Australia) and analytical grade reagents were used unless otherwise stated. Chemicals were obtained from the following sources: N-acetylcysteine (>99%), L-cysteine (>98.5%), L-cystine (>99%), L-cysteinylglycine (≥85%), disodium phosphate, N-ethylmaleimide (NEM), γ-glutamyl-cysteine (≥80%), L-glutathione (≥98%), L-glutathione disulfide (≥99%), homocysteine (≥95%), homocystine, L-methionine (≥99%), monosodium phosphate, sodium polyphosphate (+80 mesh), sodium tetraborate, trichloroacetic acid, trifluoroacetic acid and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) from Sigma-Aldrich (New South Wales, Australia); formaldehyde (37%), orthophosphoric acid (85%), potassium permanganate and sodium chloride from Chem-Supply (South Australia, Australia); sodium formate from BDH (Poole, England); ethylenediaminetetraacetic acid disodium salt (EDTA), methanol, sulfuric acid (98%) and tris(hydroxymethyl)methylamine from Merck (Victoria, Australia); formic acid from Hopkin and Williams (Essex, England); perchloric acid (70% w/v) from Univar (New South Wales, Australia); aqueous soluble starch, lead dioxide and sodium perchlorate from Ajax Finechem (New South Wales, Australia); potassium iodide 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).

Stock solutions (1 × 10⁻³ M) of N-acetylcysteine, cysteine, cystine, cysteinylglycine, γ-glutamyl-cysteine, GSH, GSSG, homocysteine, homocystine and methionine were prepared daily in deionised water that had been adjusted to pH 2.57
with trifluoroacetic acid, and diluted into the mobile phase (98% Solvent A: deionised water adjusted to pH 2.57 with trifluoroacetic acid; 2% Solvent B: methanol) as required.

The thiol selective acidic potassium permanganate reagent was prepared by dissolution of potassium permanganate \( (2.5 \times 10^{-4} \text{ M}) \) in 1% (m/v) sodium polyphosphate and adjusting to pH 3 with sulfuric acid.

The ‘standard’ acidic potassium permanganate reagent was prepared by dissolution of potassium permanganate \( (1.0 \times 10^{-3} \text{ M}) \) in 1% (m/v) sodium polyphosphate and adjusting to pH 2.5 with sulfuric acid.

The ‘enhanced’ acidic potassium permanganate reagent was prepared by dissolution of potassium permanganate \( (1.9 \times 10^{-3} \text{ M}) \) in 1% (m/v) sodium polyphosphate and adjusting to pH 2.5 with sulfuric acid and then adding sodium thiosulfate (0.6 mM), using a small volume of a 0.1 M solution.

The ‘conventional’ tris(2,2’-bipyridine)ruthenium(III) reagent was prepared by oxidising tris(2,2’-bipyridine)ruthenium(II) \( (5 \times 10^{-5} \text{ M}) \) with lead dioxide (0.2 g/100 mL) in 0.02 M sulfuric acid. Following the change in colour from orange to emerald green (approximately 5 minutes with stirring), the excess oxidant was filtered from the solution using a 0.45 μm syringe-tip filter prior to injection into the FIA manifold.

The ‘stabilised’ ruthenium perchlorate reagent was prepared as previously described [33]. This involved treating \([\text{Ru(bpy)}_3\text{Cl}_2]\) with sodium perchlorate in aqueous solution to yield the characteristic bright orange \([\text{Ru(bpy)}_3(\text{ClO}_4)_2]\) precipitate, which was collected by vacuum filtration, washed twice with ice water, and dried over phosphorus pentoxide for 24 hours. The \([\text{Ru(bpy)}_3(\text{ClO}_4)_2]\) crystals
(5 × 10⁻⁵ M) were oxidised with lead dioxide (0.2 g/100 mL) in acetonitrile containing 0.05 M perchloric acid. Following the change in colour from orange to blue-green (approximately 1 minute with stirring), the excess oxidant was filtered from the solution using a 0.45 μm syringe-tip filter prior to injection into the FIA manifold.

For use as a flowing stream of reagent, the [Ru(bipy)₃](ClO₄)₂ crystals (1 × 10⁻³ M) were oxidised with 0.2 g/100 mL lead dioxide in acetonitrile containing 0.05 M perchloric acid. Following 1 minute of vigorous mixing and the characteristic change in colour from orange to blue-green, the resultant suspension was allowed to settle prior to filtration through an in-line filter (consisting of a small Pasteur pipette packed tightly with glass wool).

Soluble manganese(IV) was prepared as previously described [46], based on the method of Jáky and Zrinyi [45]. This involved the reduction of potassium permanganate using excess sodium formate to yield the characteristic dark brown manganese dioxide precipitate which was collected by vacuum filtration (GF/A Whatman, England). After rinsing with deionised water, the freshly precipitated wet manganese dioxide (1.2 g) was added to 1.0 L of orthophosphoric acid (3.0 M) and ultrasonicated for 30 min. The colloid was then heated for 1 hour (80 °C) with stirring, and then cooled to room temperature. The concentration was determined by iodometric titration as described by Vogel [218]. The stock manganese(IV) reagent was diluted daily to the required concentration (5 × 10⁻⁴ M) using orthophosphoric acid (3.0 M). Formaldehyde was filtered and diluted to the required concentration with deionised water.
2.2 Flow injection analysis

The FIA manifold was constructed from a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Victoria, Australia) with bridged PVC pump tubing (1.02 mm i.d.; DKSH, Queensland, Australia), black manifold tubing (0.76 mm i.d., Global FIA, WA, USA) and six-port injection valve (Vici 04 W-0192L, Valco Instruments, Texas, USA) equipped with a 70 μL sample loop. A GloCel chemiluminescence detector (Global FIA) with flow-cell D (dual-inlet serpentine) and extended range photomultiplier module (Electron Tubes model P30A-05; ETP, New South Wales, Australia) was used for the permanganate and manganese(IV) experiments, whilst an in-house fabricated detector containing an aluminium-backed coiled-tubing flow-cell and extended-range photomultiplier module within a light-tight housing was used for the tris(2,2′-bipyridine)ruthenium(III) experiments. All tubing entering and exiting the detectors was black PTFE (0.76 mm i.d). The output signal from the photomultiplier module was recorded using ‘e-corder 410’ data acquisition software (eDAQ, New South Wales, Australia).

2.2.1 Acidic potassium permanganate

The analytes \((1 \times 10^{-5} \text{ M})\) were injected (70 μL) into a 100% aqueous carrier stream (2.5 mL/min or 3.5 mL/min) that merged with the acidic potassium permanganate reagent (2.5 mL/min or 3.5 mL/min) within the reaction channel of the flow-cell (Figure 44).
2.2.2 Tris(2,2'-bipyridine)ruthenium(III)

**Manifold 1:** A continuously flowing analyte stream (5 × 10⁻⁶ M; 3.5 mL/min) was merged with a sodium tetraborate buffer (0.1 M, pH 10; 3.5 mL/min) at a T-piece located 15 cm prior to the flow-cell inlet. The ruthenium reagents were injected (70 μL) into a 100% aqueous carrier stream (deionised water; 3.5 mL/min) that merged with the combined analyte-buffer solution just prior to entry into the flow-cell (Figure 45).

**Manifold 2:** The analytes (1 × 10⁻⁵ M) were each injected (70 μL) into a 100% aqueous carrier stream (formic acid pH 2.8; 1 mL/min) that merged with a phosphate buffer (0.04 M; pH 8.0; 1 mL/min) at a T-piece located 15 cm prior to the entrance of the flow-cell. A continuously flowing stream of the ruthenium reagent
(1 \times 10^{-3} \text{ M}; 2 \text{ mL/min}) then merged with the combined analyte-buffer solution just prior to entry into the flow-cell (Figure 46).

![Figure 46. Schematic of the FIA manifold used with the tris(2,2’-bipyridine)ruthenium(III) reagent as a flowing stream.](image)

2.2.3 **Soluble manganese(IV)**

The analytes (1 \times 10^{-5} \text{ M}) were each injected (70 \mu\text{L}) into a 100% aqueous carrier stream (deionised water; 3.5 mL/min) that merged with a flowing stream of 2.0 M formaldehyde (used as an enhancer to significantly improve the emission intensity; 3.5 mL/min) at a T-piece located 22 cm prior to the flow-through detector inlet. A continuously flowing stream of the manganese(IV) reagent (3.5 mL/min) merged with the combined analyte-formaldehyde solution in the reaction channel of the flow-cell (Figure 47).

![Figure 47. Schematic of the FIA manifold used for manganese(IV) chemiluminescence measurements.](image)
2.3 Chemiluminescence spectra

A Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Victoria, Australia) with R928 photomultiplier tube (Hamamatsu, Japan) was operated in Bio/Chemiluminescence mode. A three-line continuous flow manifold was used to merge the GSH or GSSG solution (1 × 10⁻⁴ M; 1.0 mL/min) with formaldehyde (2.0 M; 1.0 mL/min), and then with the manganese(IV) reagent (5 × 10⁻⁴ M in 3.0 M orthophosphoric acid; 1.0 mL/min), and propel the reacting mixture through a coiled PTFE flow-cell (200 µL, 0.8 mm i.d.) that was mounted against the emission window of the spectrophotometer. Final spectra were an average of 20 scans (1000 ms gate time, 1 nm data interval, 20 nm band pass, PMT: 800 V) and corrected as previously described [219].

2.4 High performance liquid chromatography

Chromatographic analysis was carried out on an Agilent Technologies 1200 series liquid chromatography system, equipped with a quaternary pump, solvent degasser system and autosampler (Agilent Technologies, Victoria, Australia), using an Alltech Alltima C18 column (250 mm × 4.6 mm i.d., 5 µm) at room temperature, with an injection volume of 100 µL and a flow rate of 1 mL/min. Isocratic elution was performed with 98% Solvent A: deionised water adjusted to pH 2.57 with trifluoroacetic acid and 2% Solvent B: methanol. A Hewlett-Packard analogue 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 manganese(IV) chemiluminescence measurements, the column eluate (1.0 mL/min) and a formaldehyde carrier (2.0 M; 1.0 mL/min) were merged at a
T-piece located 22 cm from the entrance of the detector. This stream was then combined with the manganese(IV) reagent \(5 \times 10^{-4} \text{ M in } 3.0 \text{ M orthophosphoric acid; } 1.0 \text{ mL/min}\) in the detector employed for FIA as described above. A peristaltic pump was used to deliver the reagents (Figure 48).

![Diagram](image)

**Figure 48.** Schematic of the HPLC manifold employed for manganese(IV) chemiluminescence measurements.

For acidic potassium permanganate chemiluminescence measurements, the column eluate (1.0 mL/min) and reagent (2.5 mL/min) merged at a T-piece and the light emitted from the reacting mixture was detected with a custom built flow-through chemiluminometer, which consisted of a coiled flow-cell comprising of 0.8 mm i.d. PTFE tubing (DKSH), mounted flush against the window of Electron Tubes photomultiplier tube (model 9878SB, ETP) set at a constant voltage of 900 V from a stable power supply (PM20D, ETP) via a voltage divider (C611, ETP). The flow-cell, photomultiplier tube and voltage divider were encased in light-tight housing (Figure 49).
2.5 Sample collection and analysis

The method of Stempak et al. [120] was followed when collecting and processing blood samples. Venous blood samples (5 mL) were drawn from twelve healthy, non-fasting adult volunteers into ethylenediaminetetraacetic acid disodium salt (EDTA) lined blood tubes (Becton and Dixon, UK) and immediately placed on ice. Aliquots of ice-cold perchloric acid (500 μL, 15% (v/v) containing 2.0 mM EDTA) were pipetted into 1.5 mL Eppendorf tubes, followed by whole blood (500 μL). The samples were then vortexed and incubated on ice for 10 minutes to completely precipitate blood proteins. The acidic suspensions were then centrifuged at 13000 rpm for 15 minutes at 4 °C, and immediately analysed. For analysis, 10 μL of the acidic supernatant was mixed with 990 μL of mobile phase (98% Solvent A: deionised water adjusted to pH 2.57 with trifluoroacetic acid; 2% Solvent B: methanol), vortexed and filtered with a 0.2 μm cellulose acetate filter, prior to injection onto the HPLC column.
3. Results and Discussion

3.1 Acidic potassium permanganate chemiluminescence detection

Li and co-workers previously reported the determination of thiols such as cysteine, GSH, N-acetylcysteine and captopril based on the sensitised chemiluminescence reaction of acidic potassium permanganate and quinine [123]. This system contains two light producing pathways: (i) the reduction of permanganate to form an excited state manganese(II) species, and (ii) the oxidation of the thiol compounds to generate an excited intermediate capable of transferring energy to the quinine fluorophore [13, 202].

This approach was improved by McDermott and co-workers via the substitution of quinine with sodium polyphosphate to promote the manganese(II) pathway [18]. Removing quinine eliminated the background emission from the reaction between the sensitiser and oxidant, enabled post-column chemiluminescence detection of GSH using a single reagent solution and provided greater sensitivity (the limit of detection was $5 \times 10^{-7}$ M, compared to $6.5 \times 10^{-6}$ reported by Li et al.[123]).

The selectivity of this permanganate reagent for a wider range of biologically significant thiols and disulfides (cysteine, cystine, GSH, GSSG, homocysteine and homocystine) was evaluated using FIA methodology. Analyte standard solutions ($1 \times 10^{-5}$ M) were injected into a stream of aqueous formic acid (pH 2.8; 2.5 mL/min) which merged with the permanganate reagent ($2.5 \times 10^{-4}$ M in 1% (m/v) sodium polyphosphate solution, adjusted to pH 3 with sulfuric acid; 2.5 mL/min) within the reaction channel of the flow-cell. Significant chemiluminescence signals were obtained for the three thiols, a negligible emission for cystine (which may have resulted from a minor breakdown of the disulfide to its corresponding thiol; less than
0.6% relative to cysteine), and no response for either GSSG or homocystine (Figure 50).

![Diagram showing chemiluminescence intensity for different analytes.]

**Figure 50.** Relative chemiluminescence intensities (peak heights) obtained for the oxidation of biologically significant thiols and disulfides \((1 \times 10^{-7} \text{ M})\) with a permanganate reagent optimised for the detection of thiols. Columns: cysteine (CYS), cystine (CYSS), glutathione (GSH), glutathione disulfide (GSSG), homocysteine (HCYS) and homocystine (HCYSS).

As demonstrated in chapter three, considerable increases in reaction rate and chemiluminescence intensity can be obtained by a preliminary partial reduction of permanganate to create a stable, relatively high concentration of manganese(III) in permanganate reagents [205, 213]. Therefore the thiols and disulfides were also screened with a standard permanganate reagent and the enhanced reagent.

Each of the analytes generated a response with both of the permanganate reagents. On average, the signals obtained from the thiols were 17-fold more intense than their corresponding disulfide using the standard permanganate reagent (Figure 51, blue columns). In agreement with the results presented in chapter three, the enhanced reagent provided increases in chemiluminescence intensity for the six analytes.
(1-2 fold for the thiols and 2.3-4.2 fold for the disulfides, Figure 51 pink columns). Although there are very few methodologies capable of directly detecting thiols and disulfides, the sensitivity achieved using the standard or enhanced permanganate reagents was insufficient for transfer of these approaches to post-column chemiluminescence detection.

![Graph](image)

**Figure 51.** Relative chemiluminescence intensities (peak heights) obtained for the oxidation of biologically significant thiols and disulfides (1 × 10−5 M) with (i) standard permanganate (blue columns) and (ii) the enhanced permanganate reagent (pink columns). Columns: cysteine (CYS), cystine (CYSS), glutathione (GSH), glutathione disulfide (GSSG), homocysteine (HCYS) and homocystine (HCYSS).

### 3.2 Tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection

As previously discussed, tris(2,2'-bipyridine)ruthenium(III) has been used extensively for the determination of various analytes containing an amine moiety, with tertiary substituted amines generally eliciting the largest responses, followed by secondary and then primary [30, 31, 38, 39]. Despite this, several studies have shown that an alkaline reaction environment can promote analytically useful signals from analytes containing secondary amines [23, 38, 220, 221]. The aforementioned thiols
and disulfides possess both primary and secondary amine functionalities, and as such, tris(2,2'-bipyridine)ruthenium(III) chemiluminescence holds the potential for direct detection of these biologically important analytes. Initial experiments were conducted with a conventional tris(2,2'-bipyridine)ruthenium(III) reagent as described by Costin and co-workers [23]. Using a three-line FIA manifold (Figure 45), a continuously flowing analyte stream \((5 \times 10^{-6} \text{ M}; 3.5 \text{ mL/min})\) was merged with a sodium tetraborate buffer \((0.1 \text{ M}, \text{pH} 10; 3.5 \text{ mL/min})\) and then combined with a deionised water carrier \((3.5 \text{ mL/min})\) immediately prior to entering the flow-cell. The reagent \((5 \times 10^{-6} \text{ M})\) was injected into the carrier stream. The relatively large signal obtained for homocystine (Figure 52) initially suggested that this reagent had the potential to determine biologically significant disulfides. However the response for GSSG was less than 10% relative to homocystine, and the other analytes produced emissions lower than the blank.

![Figure 52](image)

**Figure 52.** Relative chemiluminescence intensities (peak heights) obtained for the oxidation of biologically significant thiols and disulfides \((5 \times 10^{-6} \text{ M})\) with the conventional tris(2,2'-bipyridine)ruthenium(III) reagent \((5 \times 10^{-5} \text{ M})\). Columns: cysteine (CYS), cystine (CYSS), glutathione (GSH), glutathione disulfide (GSSG), homocysteine (HCYS) and homocysteine (HCYSS).
A longstanding weakness of tris(2,2′-bipyridine)ruthenium(III) as a chemiluminescence reagent has been its limited stability in aqueous solution, particularly when employing off-line methods of preparation and/or alkaline conditions [1, 5, 30, 31, 33-36]. To address this issue, an exceedingly stable reagent developed by McDermott and co-workers [33] was prepared by oxidising [Ru(bipy)₃](ClO₄)₂ in acetonitrile containing 0.05 M perchloric acid. This reagent was injected into the same three-line FIA manifold as described above. The stabilised reagent was found to exhibit similar selectivity to that of the conventional reagent, however in this instance, the response for cystine was marginally larger than the blank (despite being only 1% relative to that of homocystine, Figure 53).

Figure 53. Relative chemiluminescence intensities (peak heights) obtained for the oxidation of biologically significant thiols and disulfides \((5 \times 10^{-6} \text{ M})\) with the stabilised tris(2,2′-bipyridine)ruthenium(III) reagent \((5 \times 10^{-3} \text{ M})\). Columns: cysteine (CYS), cystine (CYSS), glutathione (GSH), glutathione disulfide (GSSG), homocysteine (HCYS) and homocystine (HCYSS).
Previous work undertaken by McDermott to optimise the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent to derive the greatest response for thiols and disulfides focused solely on the determination of GSH and GSSG [222]. The selectivity of these conditions for a wider range of thiols and disulfides was evaluated by injecting analyte standard solutions (1 × 10^{-5} M) into a stream of aqueous formic acid (pH 2.8; 1.0 mL/min) which merged with a phosphate buffer (0.04 M; pH 8.0; 1.0 mL/min) at a T-piece located 15 cm prior to the entrance of the flow-cell (Figure 46). A continuously flowing stream of the reagent (1 × 10^{-3} M; 2.0 mL/min) then merged with the combined analyte-buffer solution just prior to entry into the flow-cell. Under these conditions, each of the thiols produced emissions bigger than the blank (Figure 54). Homocysteine produced the dominant response of the three disulfides, and no light was detected from the oxidation of cystine.

![Figure 54](image)

**Figure 54.** Relative chemiluminescence intensities (peak heights) obtained for the oxidation of biologically significant thiols and disulfides (1 × 10^{-5} M) with the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent (1 × 10^{-3} M). Columns: cysteine (CYS), cystine (CYSS), glutathione (GSH), glutathione disulfide (GSSG), homocysteine (HCYS) and homocystine (HCYSS).
3.3 Manganese(IV) chemiluminescence detection

As outlined in the review by Brown et al. [43] only a small number of analytical applications of manganese(IV) chemiluminescence have been reported to-date. Of these, only one manuscript describes the use of manganese(IV) for post-column chemiluminescence detection, where a mixture of six opiate alkaloid standards were determined following HPLC separation with a monolithic column [46]. Owing to the limited number of studies, little is known regarding the relationship between analyte structure and the chemiluminescence response with this reagent [43]. Moreover, there have been no published reports of manganese(IV) chemiluminescence emission upon reaction with thiols or disulfides.

Previous work by Brown et al. found that the emission intensities for various analytes were significantly enhanced by the addition of formaldehyde [46]. Therefore, the ability of the thiols and disulfides to provide analytically useful signals upon reaction with the manganese(IV) reagent was examined using a three-line FIA manifold. The analytes ($1 \times 10^{-5}$ M) were each injected (70 μL) into a 100% aqueous carrier stream (1 mL/min) that merged with a flowing stream of 2.0 M formaldehyde (1.0 mL/min) at a T-piece located 22 cm prior to the flow-through detector inlet. The manganese(IV) reagent (1.0 mL/min) was pumped into the second inlet and merged with the combined analyte-formaldehyde solution within the serpentine reaction channel of the flow-cell. Strong signals were recorded for each of the analytes (Figure 55).
Using the aforementioned analytes, a series of univariate searches were performed on flow rate and the concentration of formaldehyde. A 2.0 M formaldehyde solution and flow rate of 1.0 mL/min per line were found to afford the greatest chemiluminescence response for each of the analytes and were used for all further experiments. The percentage of organic modifier (methanol or acetonitrile) added to the aqueous carrier stream (representing the HPLC mobile phase) was found to have a critical effect on manganese(IV) chemiluminescence. For example, the addition of only 1% acetonitrile resulted in an average 70% decrease in signal intensity for GSH (Figure 56, blue columns) and GSSG (Figure 56, pink columns). In contrast, adding methanol (in 1% increments to 5%) did not significantly alter the response.
(Figure 56). Therefore, use of acetonitrile in combination with the manganese(IV) reagent is not recommended.

![Graph showing effect of organic modifier on chemiluminescence intensity](image)

**Figure 56.** Effect of the percentage of organic modifier on the relative chemiluminescence intensities (peak heights) obtained for the oxidation of response of GSH (blue columns) and GSSG (pink columns, $1 \times 10^{-5}$ M) with manganese(IV) ($5 \times 10^{-4}$ M).

Using the optimised parameters, a wider range of biologically important thiols and disulfides (cystine, cysteine, homocystine, homocysteine, GSH, GSSG, N-acetylcysteine, cysteinylglycine, $\gamma$-glutamyl-cysteine, and methionine) were screened with the manganese(IV) reagent. All of the analytes elicited a response, demonstrating the potential of manganese(IV) chemiluminescence for the detection of both thiols and disulfides (Figure 57).
Figure 57. Relative chemiluminescence intensities (peak heights) obtained for the oxidation of biologically significant thiols and disulfides \((1 \times 10^{-5} \text{ M})\) with manganese(IV) \((5 \times 10^{-4} \text{ M})\). Columns: cysteinylglycine (CYS-GLY), cysteine (CYS), cystine (CYSS), glutathione (GSH), glutathione disulfide (GSSG), \(\gamma\)-glutamylcysteine (\(\gamma\)-GLU-CYS), homocysteine (HCYS), homocysteine (HCYSS), methionine (MET) and \(N\)-acetylcysteine (NACYS).

The chemiluminescence spectra (corrected for the detector response and monochromator transmission [202]) for the oxidation of a thiol (GSH) and a disulfide (GSSG) both contained a single broad band with a maximum at 734 ± 5 nm, which matched the characteristic emission observed from other reactions with manganese-based reagents [13, 16, 43, 48], attributed to an electronically excited manganese(II) species [17] (Figure 58).
Figure 58. (i) Uncorrected and (ii) corrected chemiluminescence spectra for the reactions between manganese(IV) ($5 \times 10^{-4}$ M) and glutathione (GSH, $1 \times 10^{-3}$ M) or glutathione disulfide (GSSG, $1 \times 10^{-4}$ M), using formaldehyde (2.0 M) as an enhancer.
3.3.1 Separation conditions and analytical figures of merit

As the vast majority of techniques for the detection of biological thiols and disulfides employ pre-column derivatisation, very few involve separation of these molecules in their native forms [84, 85, 87, 106, 108-110]. However, from these accounts, satisfactory separation has been achieved using reverse phase HPLC with an almost 100% aqueous isocratic solvent system [118-120]. A mobile phase consisting of 98% deionised water adjusted to pH 2.57 with trifluoroacetic acid (similar to that used by Khan et al. [118] and Pelletier et al. [119]) was initially examined for the separation of a mixture containing seven routinely measured thiols and disulfides. All of the analytes were separated within 35 minutes (Figure 59i) with unnecessarily high resolution between N-acetylcysteine and GSSG. The separation was tunable by simply adjusting the amount of methanol present in the mobile phase by 1% increments, with the largest effects on retention time observed for N-acetylcysteine and GSSG (co-elution of these peaks occurred with 3% methanol; Figure 59iv). The optimal mobile phase composition consisted of 98% deionised water adjusted to pH 2.57 with trifluoroacetic acid and 2% methanol. These conditions were found to provide a resolution of 1.3 between cysteine and cystine and baseline separation for all other analytes within 20 minutes (Figure 59iii).
Figure 59. Separation of a mixture containing thiols and disulfides ($1 \times 10^{-5}$ M) using various percentages of methanol in the mobile phase (deionised water adjusted to pH 2.57 with trifluoroacetic acid): (i) 0%, (ii) 1%, (iii) 2% and (iv) 3%. Peaks: cystine (CYSS), cysteine (CYS), homocysteine (HCYS), homocystine (HCYSS), glutathione (GSH), N-acetylcysteine (NACYS), and glutathione disulfide (GSSG).
Furthermore, to highlight the advantage of this procedure in comparison to the previously reported chemiluminescence approach [18], the same mixture was subjected to an identical separation but detected using acidic potassium permanganate (Figure 60ii). Whilst both reagents can be used to detect thiols, the manganese(IV) reagent also enabled the direct detection of the disulfides (Figure 60i).

**Figure 60.** Separation of a mixture containing thiols and disulfides \((1 \times 10^{-5} \text{ M})\) using either (i) manganese(IV) \((5 \times 10^{-4} \text{ M})\) or (ii) acidic potassium permanganate \((2.5 \times 10^{-4} \text{ M})\) chemiluminescence detection.
The procedure was evaluated in terms of linearity, sensitivity and precision (Table 7). Calibration curves were constructed for each analyte using twelve standards prepared over the range of $3 \times 10^{-8}$ M to $5 \times 10^{-5}$ M. As can be seen from the correlation coefficients ($R^2$, Table 7), all thiols and disulfides exhibited a highly linear relationship between signal (peak area) and concentration over their respective dynamic range. The limit of detection (LOD, defined as a signal-to-noise ratio of 3), for all analytes was in the range of $5 \times 10^{-8}$ M to $1 \times 10^{-7}$ M, which is comparable to other HPLC techniques for the detection of thiols and disulfides without derivatisation [87, 106, 110, 117, 118]. The precision of repeated injections was excellent for all analytes; relative standard deviations were less than 0.3% for retention times and 2% for peak areas.

**Table 7.** Analytical figures of merit obtained for the thiols and disulfides using manganese(IV) ($5 \times 10^{-8}$ M) chemiluminescence detection.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention</th>
<th>$R^2$</th>
<th>Linear range (µM)</th>
<th>R.S.D. (%)</th>
<th>LOD (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
<td>R.S.D. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cystine</td>
<td>2.6</td>
<td>0.1</td>
<td>0.9881</td>
<td>0.05 - 10</td>
<td>1.3</td>
</tr>
<tr>
<td>cysteine</td>
<td>3.2</td>
<td>0.1</td>
<td>0.9958</td>
<td>0.05 - 10</td>
<td>1.1</td>
</tr>
<tr>
<td>homocysteine</td>
<td>4.3</td>
<td>0.1</td>
<td>0.9985</td>
<td>0.05 - 7</td>
<td>0.8</td>
</tr>
<tr>
<td>homocystine</td>
<td>5.5</td>
<td>0.2</td>
<td>0.9978</td>
<td>0.05 - 7</td>
<td>1.4</td>
</tr>
<tr>
<td>GSH</td>
<td>7.0</td>
<td>0.1</td>
<td>0.9982</td>
<td>0.07 - 10</td>
<td>1.4</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>14.1</td>
<td>0.1</td>
<td>0.9990</td>
<td>0.07 - 10</td>
<td>1.1</td>
</tr>
<tr>
<td>GSSG</td>
<td>18.2</td>
<td>0.3</td>
<td>0.9982</td>
<td>0.1 - 10</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*$n = 10$

**3.3.2 Determination of GSH and GSSG in whole blood**

As previously noted, quantifying the molar ratio of GSH to its dimer, GSSG, is one of the most important indicators of cellular health [85, 97-99, 112]. Although these
biomarkers are ubiquitous in all human organs, it has been suggested that their concentrations in blood may reflect the redox status of other less accessible tissues and hence provide a useful indicator of a whole subject’s oxidative status [88, 114, 115, 223, 224]. However, the sample processing required in most procedures for the determination of GSH and GSSG in blood (i.e. thiol blocking and/or derivatisation, disulfide bond reduction, sample pH neutralisation, etc.) can lead to erroneous measurements [87, 114, 115, 224]. Therefore, the use of HPLC with manganese(IV) chemiluminescence detection was explored for the determination of GSH and GSSG in whole blood, where samples were simply deproteinised, centrifuged and diluted prior to analysis.

The deproteinisation of physiological fluids prior to GSH and GSSG determination is an indispensable step [84, 85, 87, 106, 107, 110]. Stempak et al. [120] examined a range of commonly employed acids for the deproteinisation of blood and found that combining samples (1:1) with perchloric acid (15% (v/v) containing 2.0 mM EDTA) prior to storage at -80 °C afforded the greatest GSH and GSSG stability. However, Rossi et al. [114] noted that deproteinisation with perchloric acid or trichloroacetic acid significantly altered the GSH/GSSG ratio in blood. The methods of sample preparation described by both Stempak et al. [120] and Rossi et al. [114] were examined using two blood samples, prepared in triplicate, with and without the initial introduction of N-ethylmaleimide (NEM), which reacts with the free thiol of GSH to prevent unwanted oxidation.

Based on the deproteinisation procedure described by Rossi et al. [114], a whole blood sample was divided into two aliquots. The first aliquot (370 μL) was combined with a solution of 1.5 g/L EDTA and 250 mM sodium chloride (40 μL) and then trichloroacetic acid (600 g/L, 77 μL), centrifuged and diluted 38-fold into the mobile
phase, immediately prior to analysis using HPLC with manganese(IV) chemiluminescence detection (Figure 61i; black trace). The second aliquot was mixed with a solution of 1.5 g/L EDTA and 0.5 M NEM (40 µL) and trichloroacetic acid (600 g/L; 77 µL) [114], centrifuged, diluted 38-fold into the mobile phase, and analysed (Figure 61i; pink trace). The disappearance of the peak at 7.0 minutes confirmed the complete reaction of GSH with NEM. A difference (55-62%) in the GSSG peak (18.2 min) was observed for samples with and without NEM, suggesting that significant oxidation of GSH had occurred during the deproteinisation procedure. However, the change was much lower than that reported by Rossi et al. [114], possibly arising from additional error introduced during their subsequent liquid-liquid extraction, analyte derivatisation (pH 10.8, room temperature, 2 hr) and re-acidification, prior to analysis.

To replicate the sample preparation conditions of Stempak et al. [120], a whole blood sample was divided into two aliquots, one of which was combined (1:1) with perchloric acid (15% (v/v) containing 2.0 mM EDTA), centrifuged and diluted 25-fold into the mobile phase, immediately prior to analysis (Figure 61ii; black trace). The second aliquot was mixed with NEM (3.6 mM) and a Tris-HCl buffer (0.1 M; pH 8.0) in a 2:1:1 ratio. It has previously been shown that under these conditions, NEM reacts immediately and completely with GSH to form a thioether derivative [18]. This solution was then acidified by combining with an equal volume of perchloric acid (15% (v/v) containing 2.0 mM EDTA), centrifuged, and diluted 12.5-fold into the mobile phase and analysed (Figure 61ii; pink trace). The disappearance of the GSH peak again confirmed complete reaction with NEM. In contrast to the results shown in Figure 61i, only a small change (less than 6%) in the
peak area for GSSG was observed, indicating that this deproteinisation procedure does not result in significant oxidation of GSH to GSSG in whole blood samples.

**Figure 61.** Typical manganese(IV) chemiluminescence chromatograms obtained for the analysis of whole blood, using the deproteinisation procedures of (i) Rossi and co-workers and (ii) Stempak and co-workers. Pink and black traces show sample preparation with and without the addition of NEM.
The method was then used to determine GSH and GSSG in whole blood from twelve healthy volunteers (six men and six women, 21-31 years old). The results of the study are presented in Table 8, with values obtained for GSH (723–1260 μM) and GSSG (120–191 μM) within the range previously reported by numerous other researchers for healthy subjects of similar age [223-227].

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (years)</th>
<th>GSH (μM/L)</th>
<th>GSSG (μM/L)</th>
<th>GSH/GSSG</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>R.S.D. (%)</td>
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</tr>
<tr>
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<td>F</td>
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\[n = 3\]
4. Conclusions

This chapter presented a systematic evaluation of the relative chemiluminescence responses from several biologically significant thiols and disulfides with conventional and new adaptations of permanganate and tris(2,2'-bipyridine)ruthenium(III) reagents, in addition to a manganese(IV) colloid. This colloid provided a simple and sensitive approach for the direct detection of thiols and disulfides using both FIA and HPLC methodologies. A mixture of seven thiols and disulfides (cysteine, cystine, homocysteine, homocystine, glutathione (GSH), glutathione disulfide (GSSG) and N-acetylcysteine) in their native forms were separated within twenty minutes. Detection limits for these analytes ranged from $5 \times 10^{-8}$ M to $1 \times 10^{-7}$ M, and the precision for retention times and peak areas was excellent, with relative standard deviations of less than 0.3% and 2%, respectively. This procedure was successfully applied to the determination of two key biomarkers of oxidative stress, GSH and GSSG in whole blood taken from twelve human subjects. Preliminary experiments showed that preparation of samples via centrifugation, deproteinisation (using perchloric acid (15% (v/v) containing 2.0 mM EDTA)) and dilution was simple and did not result in significant GSH oxidation. Concentrations of GSH and GSSG in the blood samples were comparable to other reports. The findings of this chapter have resulted in two publications [228, 229].
CHAPTER FIVE

Precision milled chemiluminescence flow-cells for flow injection analysis and high performance liquid chromatography

- Introduction
- Experimental
- Results and discussion
- Conclusion
CHAPTER FIVE

1. Introduction

The analytical utility of liquid-phase chemiluminescence detection is dependent on the reproducibility of solution mixing, reaction kinetics and the proportion of the transient emission exposed to a suitable photodetector. As described in the preceding chapters, greater sensitivity can be obtained by combining exceedingly stable chemiluminescence reagents that enable greater control of reaction rates with machined flow-cells that more efficiently mix the reacting solutions. This work has further demonstrated the need to refine several key design attributes of chemiluminescence flow-cells, such as the proximity of the confluence point to the detection zone in relation to the rate of the light-producing reaction [73, 77], the use of reflective surfaces to direct more of the emitted light towards the photodetector [234, 235], and increased mixing efficiency via greater deviation in the flow path [73, 81].

To address some of the aforementioned issues, flow-cells have previously been constructed by engraving or machining channels into Perspex [167, 168], Acetal [169] or Teflon [73] and sealing the channels with transparent films or plates. Similarly, chemiluminescence detection has been incorporated into microfluidic devices fabricated from silicon, glass or polymer materials [230]. In these systems, solutions are often merged in T- or Y-shaped channels with a linear detection zone [165, 166, 231], which exposes only a very small volume of solution to the relatively large photodetector window. Microscope optics have been used to partially alleviate this limitation [232]. Alternatively, spiral [161] or meandering [163, 170, 233] channels have been used to improve mixing and increase the volume of solution within the detection zone. In spite of these developments, there has been very little
optimisation of chemiluminescence flow-cell or microfluidic-reactor configuration, which has often been limited by fabrication capabilities.

Other flow-cell designs have also been explored [73, 77, 81, 83, 234-238], such as the fountain [83], sandwich [77], liquid core waveguide [234], bundle [81], vortex [236], and droplet [237] flow-cells, but as yet none have attained widespread acceptance, generally due to the complexity of fabrication, current lack of evidence for significant advantages over the coiled-tubing approach, and/or limited applicability.

This chapter details the use of computer-aided design and high-precision machining technology to create novel flow-cells with integrated confluence points and reaction zones for highly sensitive detection using relatively fast chemiluminescence reactions. These new designs were investigated in direct comparison with the conventional coiled-tubing approach and several previously described flow-cells to determine their effect on the generation and transmission of light to the photodetector.
2. Experimental

2.1 Chemicals and reagents

Deionised water (Continental Water Systems, Victoria, Australia) and analytical grade reagents were used unless otherwise stated. Chemicals were obtained from the following sources: amoxicillin, epinephrine hydrochloride, fenoterol hydrobromide, isoprenaline hydrochloride, luminol, metaproterenol hemisulfate salt, octopamine, sodium polyphosphate (+80 mesh), sodium thiosulfate, synerpine, trifluoroacetic acid, tyramine and vanilmandelic acid from Sigma-Aldrich (NSW, Australia); N-methyltyramine from CSIRO Animal Health Laboratories (Victoria, Australia); salbutamol hemisulfate from Fluka (NSW, Australia); morphine from GlaxoSmithKline (Victoria, Australia); potassium permanganate from Chem-Supply (SA, Australia); methanol and sulfuric acid from Merck (Victoria, Australia); potassium ferricyanide and sodium hydroxide from Ajax (NSW, Australia). Hordenine was synthesised as previously described [22].

Morphine (1 × 10⁻³ M) was prepared in acidified deionised water and sonicated to aid dissolution. Vanilmandelic acid (1 × 10⁻³ M) was prepared by dissolving the solid in sodium hydroxide (0.1 M) and diluting in deionised water as required. The Citrus aurantium compounds (hordenine, N-methyltyramine, octopamine, synerpine and tyramine, 1 × 10⁻³ M) were prepared in deionised water and diluted into the HPLC mobile phase (98% Solvent A: deionised water adjusted to pH 2.15 with trifluoroacetic acid; 2% Solvent B: methanol) as required. The β-adrenergic agonists (epinephrine, fenoterol, isoprenaline, metaproterenol and salbutamol, 1 × 10⁻³ M) were prepared and diluted in deionised water adjusted to pH 2.5 with trifluoroacetic acid.
The ‘standard’ acidic potassium permanganate reagent was prepared by dissolution of potassium permanganate (1 × 10⁻³ M) in 1% (m/v) sodium polyphosphate and adjusting to pH 2.5 with sulfuric acid.

The ‘enhanced’ acidic potassium permanganate reagent was prepared by dissolution of potassium permanganate (1.9 × 10⁻³ M) in 1% (m/v) sodium polyphosphate and adjusting to pH 2.5 with sulfuric acid and then adding sodium thiosulfate (0.6 or 1.0 mM), using a small volume of a 0.1 M solution.

The luminol reagent (3.5 × 10⁻⁶ M) was prepared by dissolution of the solid in deionised water, following the addition of a small volume of aqueous sodium hydroxide (1.0 M), whilst potassium hexacyanoferrate(III) was prepared by dissolution of potassium ferricyanide (3 × 10⁻⁴ M) in sodium hydroxide (0.35 M).

To obtain intense luminol chemiluminescence suitable for visual examination, the luminol reagent was prepared by dissolution of luminol (1.1 × 10⁻³ M) in sodium hydroxide (0.1 M), whilst potassium hexacyanoferrate(III) (potassium ferricyanide, 3 × 10⁻² M) was prepared in deionised water containing 4% (v/v) hydrogen peroxide [173].

2.2 Flow-cells

Flow-cell A was constructed by mounting a tight coil (28 mm diameter) of transparent PTFE tubing (0.8 mm i.d.; DKS) onto a thin metal sheet (35 × 60 mm). The tubing at the centre of the coil passed through a slit in the sheet and was connected to a barbed plastic T-piece by slipping silicone tubing (1.02 mm i.d.; DKS) over both the tubing and the fitting. The distance from the confluence point to the beginning of the coil was 1 cm (approximately 5 µL).
Flow-cell C was a Teflon disk with a machined serpentine channel (0.76 mm width × 0.89 mm depth), contained within a commercially available GloCel chemiluminescence detector (as described in chapter 2, section 2.4).

Flow-cell D was a modified version of flow-cell C, containing two solution inlets at the centre of the Teflon disk and an appropriately adjusted back plate.

Flow-cells G and H were identical to flow-cells E and F, except that the disks were constructed from Teflon impregnated with 25% glass microspheres to improve the transmission of light to the photomultiplier tube.

Several novel flow-cells with integrated confluence points and reaction channels designed for efficient mixing of fast chemiluminescence systems were constructed by machining opposing sides of a polymer chip and sealing the channels with transparent epoxy-acetate films. Prior to fabrication, 3D models of the flow-cells were drawn using Autodesk Inventor (Autodesk Inc., San Rafael, US), with the exception that the sinusoidal reaction zone was calculated using Visual Basic (Microsoft) and imported into AutoCAD (AutoDesk). Each flow-cell had dimensions of 46 × 57 × 6 mm and contained a 24-28 mm diameter reaction zone – such as the spiral-configuration (Figure 62i) – and a confluence point located below the central inlet of the reaction zone (Figure 62ii). EdgeCAM software (EdgeCam UK, Reading, UK) was used to convert the 3D models into machine code for the CNC precision milling machine (Datron CAT3D M6, Datron Technology Ltd, Milton Keynes, UK). The channels were machined into 6 mm thick sheets of polycarbonate or white acetal (both RS, Corby, UK) and sealed using a transparent epoxy-acetate film [230], which also served as the detection window on the reaction face of the chip. Solution lines were connected to the flow-cells using 062 Minstac screw-in fittings (Aviaquip, Cheltenham, Victoria, Australia).
Flow-cell L was constructed by machining a spiral channel \((0.44 \times 0.44 \text{ mm})\) into the front face of polycarbonate chip \((46 \times 57 \times 6 \text{ mm})\) and sealing the channels with a transparent epoxy-acetate film. A central entrance located above the confluence point was machined onto the back face.

Flow-cell M was identical to flow-cell L except a mirror was attached to the back face of the flow-cell to enhance the transmission of light to the photodetector.

Flow-cell N was a modification of flow-cell L, containing a \(0.7 \times 0.7 \text{ mm}\) spiral channel machined into the front face of polycarbonate chip.

Flow-cell O was identical to flow-cell N except a mirror was attached to the back face of the flow-cell to enhance the transmission of light to the photodetector.

Flow-cell P was a modification of flow-cell N containing a \(0.8 \times 0.8 \text{ mm}\) spiral channel machined into the front face of polycarbonate chip.

Flow-cell Q was identical to flow-cell P except a mirror was attached to the back face of the flow-cell to enhance the transmission of light to the photodetector.

Flow-cell R was a modification of flow-cell N, containing a spiral channel \((0.7 \times 0.7 \text{ mm})\), machined into an opaque Acetal chip and sealed with a transparent epoxy-acetate film. The total volume of the detection zone was \(144 \mu\text{L}\).
Flow-cell S was a modification of flow-cell R, containing a sinusoidal channel configuration (0.7 × 0.7 mm), machined into an opaque Acetal chip. The total volume of the detection zone was 133 µL.

Flow-cell T was a modification of flow-cell R, containing a serpentine channel configuration (0.7 × 0.7 mm), machined into an opaque Acetal chip (Figure 63). The total volume of the detection zone was 198 µL.

![Figure 63. Flow-cell T: an opaque Acetal chip with serpentine reaction channel.](image)

Flow-cell U was identical to flow-cell T, containing a serpentine channel configuration (0.7 × 0.7 mm), machined into polycarbonate (Figure 64) and a mirror attached to the back face of the flow-cell to enhance the transmission of light to the photodetector.

![Figure 64. Flow-cell U: a polycarbonate chip with serpentine reaction channel.](image)

Flow-cell W was a novel variation to the conventional coiled-tubing design. The tubing was glued into a spiral channel machined into a polished aluminium plate.
(Figure 65). Acting as a reflector, the machined channel had a semi-spherical profile matching the radius of the tubing.

![Images of flow-cell W](image1)

**Figure 65.** Construction of flow-cell W: (i) a spiral channel machined into an aluminium plate, (ii) PTFE-PFA tubing (0.8 mm i.d.) glued into the channel.

Flow-cell X was a commercially available borosilicate chip (48 × 57 × 11 mm) containing a spiral channel (1 mm i.d.) sealed by thermal bonding (Hellma, Singapore). The confluence point was located below the central entrance to the spiral (Figure 66). Inlet and outlet ports had ¼"-28 thread for conventional fittings. The back of the cell was coated with a mirror surface.

![Image of flow-cell X](image2)

**Figure 66.** Flow-cell X: a borosilicate chip with spiral reaction channel.

Flow-cell Y was identical to flow-cell X, except that all surfaces not exposed to the PMT were covered with aluminium foil.
2.3 Flow injection analysis

The FIA manifold was constructed from a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Balwyn, Victoria, Australia) with bridged PVC pump tubing (1.02 mm i.d.; DKSH), black manifold tubing (0.76 mm i.d., Global FIA, WA, USA), and six-port injection valve (Vici 04W-0192L, Valco Instruments, Houston, Texas, USA) with 70 μL injection loop. The output signal from the photomultiplier module was captured on a chart recorder (3066 Pen Recorder, Yokogawa Electric Works, Tokyo, Japan). Peak heights were measured manually.

The analytes (1 × 10^5 M) were injected (70 μL) into a deionised water carrier stream that merged with the acidic potassium permanganate reagent within the reaction channel of the flow-cell (Figure 67). Solution flow rates were optimised over the range 0.9 mL/min to 3.5 mL/min per line, for each reaction within each flow-cell.

![Figure 67. Schematic of the FIA manifold used for acidic potassium permanganate chemiluminescence measurements.](image)

2.4 High performance liquid chromatography

Analyses were carried out on an Agilent Technologies 1200 series liquid chromatography system, equipped with a quaternary pump, solvent degasser system and autosampler (Agilent Technologies, Victoria, Australia), using a Synergi Hydro-RP 80A (250 mm × 4.6 mm i.d., 4 μm) (for the C. aurantium compounds) or
an Alltech Alltima C18 column (250 mm × 4.6 mm i.d., 5 μm) (for the β-adrenergic agonists) with an injection volume of 20 μL and a flow rate of 1 mL/min. An analogue 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 the separation of _C. aurantium_ compounds, isocratic elution was performed with 98% solvent A: deionised water adjusted to pH 2.15 with trifluoroacetic acid and 2% solvent B: methanol. The column eluate (1.0 mL/min) and acidic potassium permanganate reagent (1.0 mL/min) merged at a confluence point located immediately prior or within the detection zone of the flow-cells (Figure 68).

For the separation of β-adrenergic agonists, gradient elution was performed with deionised water adjusted to pH 2.5 with trifluoroacetic acid (solvent A) and methanol (solvent B) as follows: 0-7 min: 10% B, 7-9 min: 10-50% B, 9-10 min 50% B, 10-11 min: 50-40% B, 11-12 min: 40-10% B, 12-13 min: 10% B.

**Figure 68.** Schematic of the HPLC manifold employed for acidic potassium permanganate chemiluminescence measurements.
3. Results and discussion

3.1 Channel size

Three spiral configuration flow-cells were prepared by machining channels with a width and depth of $0.44 \times 0.44$ mm, $0.7 \times 0.7$ mm and $0.8 \times 0.8$ mm into transparent polycarbonate chips (Figure 62) and sealing with an epoxy-acetate film. The spirals comprised eight turns of the $0.7$ mm or $0.8$ mm channels or fifteen turns of the $0.44$ mm channel. The channels have cross sectional areas similar to tubing with internal diameters of $0.5$, $0.8$ and $0.9$ mm (Table 9). The glass or polymer tubing used in conventional coiled flow-cell chemiluminescence detectors typically has an internal diameter between $0.5$ and $1$ mm.

<table>
<thead>
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<th>Diameter</th>
<th>Area (mm$^2$)</th>
<th>Diameter</th>
<th>Area (mm$^2$)</th>
</tr>
</thead>
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<td>$0.5$</td>
<td>$0.196$</td>
<td>$0.44$</td>
<td>$0.194$</td>
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<td>$0.8$</td>
<td>$0.503$</td>
<td>$0.7$</td>
<td>$0.490$</td>
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<td>$0.9$</td>
<td>$0.636$</td>
<td>$0.8$</td>
<td>$0.640$</td>
</tr>
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</table>

Table 9. Comparison between cross sectional area of tubing and square channels.

These flow-cells were compared using the rapid chemiluminescence reaction of morphine with acidic potassium permanganate. Using FIA methodology, six morphine standards (between $1 \times 10^{-10}$ M and $1 \times 10^{-5}$ M) were injected into a deionised water carrier stream that merged with the permanganate reagent at the confluence point within the chips (Figure 62ii). Increasing the channel dimensions from $0.44 \times 0.44$ mm (flow-cell M) to $0.7 \times 0.7$ mm (flow-cell O) improved the chemiluminescence signal by an average of $30\%$ (Figure 69, blue columns). A further $27\%$ (average) improvement was obtained using channel dimensions of
0.8 × 0.8 mm (flow-cell Q). In both cases, the percentage differences were greater at higher morphine concentrations.

The transmission of light to the photomultiplier tube was enhanced by 36-99% (0.44 mm channel), 25-45% (0.7 mm) and 18-30% (0.8 mm) by placing a mirror against the back face of the chip to reflect light back to the detector (flow-cells N, P and R respectively, Figure 69, pink columns).

![Figure 69](image_url)

**Figure 69.** Chemiluminescence intensities (peak heights) obtained for the polycarbonate chips (blue columns) and with the addition of a backing mirror (pink columns), for the reaction of morphine \((1 \times 10^{-7} \text{ M})\) with permanganate. Black points: chemiluminescence signal divided by the cross-sectional area of the channel (from data obtained without a backing mirror).

Whilst the absolute signals were lower for the flow-cells M and N (0.44 mm channels) than those with flow-cells O and P (0.7 mm channels) or Q and R (0.8 mm channels), the ratio of signal intensity to channel cross sectional area was superior with flow-cells M and N (Figure 69, black points).
3.2 Chip material

Even with a backing mirror to enhance the transmission of light to the photomultiplier tube, the chemiluminescence signal from the polycarbonate flow-cells was lower than that obtained using the conventional coiled-tubing approach (flow-cell A). A visual inspection of the light emanating from the relatively long-lived and intense chemiluminescence reaction of luminol with hydrogen peroxide and potassium hexacyanoferrate(III) in alkaline solution within the polycarbonate flow-cells revealed that a significant portion of the light was transferred out of the sides of the chips (Figure 70).

![Figure 70](image)

*Figure 70.* Chemiluminescence from the reaction between luminol, potassium hexacyanoferrate(III) and hydrogen peroxide within a polycarbonate chip with a backing mirror (exposure time 1.3 s).

Flow-cell O (0.7 × 0.7 mm channels) was then compared to a flow-cell with identical configuration that had been machined into white acetal (flow-cell S; both were sealed with a transparent epoxy-acetate film), and flow-cell A (0.8 mm i.d.), using the reactions of morphine with permanganate, and luminol with potassium hexacyanoferrate(III) enhanced by vanilmandelic acid. Compared to the luminol system used for visual examination (Figure 70), the reaction without the presence of hydrogen peroxide produces a relatively weak emission. Vanilmandelic acid can be
determined based on its enhancing effect [177], which is most prominent in the first few seconds after the solutions are merged.

More light was transferred to the photomultiplier tube from flow-cell S than flow-cells O or A, for both chemiluminescence reactions (Figure 71). The difference could be observed visually, using the intense chemiluminescence reaction of luminol with hydrogen peroxide and potassium hexacyanoferrate(III) (Figure 72). To ensure identical chemical conditions, the reactant solutions were simultaneously pumped through both chips.

![Graph showing chemiluminescence intensities](image)

**Figure 71.** Chemiluminescence intensities (peak heights) obtained from the transparent and opaque flow-cells with identical spiral channel design (0.7 × 0.7 mm) and a coil of tubing (0.8 mm i.d.), for the reaction of morphine (1 × 10^{-7} M) with permanganate (blue columns) and the reaction of luminol with hexacyanoferrate(III) enhanced by vanilmandelic acid (1 × 10^{-6} M, pink columns).
3.3 Channel design

As demonstrated in chapter 2, the emission of light from spiral reactors can be enhanced by introducing a series of reversing turns, which has been referred to as a 'serpentine' configuration. A comparison was therefore undertaken between white acetal chips that incorporate spiral (Figure 62i) and serpentine (Figure 73) reaction zones, and a third design, in which a sinusoidal pattern with a wavelength of 2.35 mm and amplitude of 0.55 mm was imposed on the spiral channel (flow-cells S, T and U respectively). Each flow-cell had an integrated confluence point (Figure 62i) and was sealed on both sides with a transparent epoxy-acetate film.

Figure 72. Visual comparison of the emission intensity from (i) flow-cell S and (ii) flow-cell O with an identical channel design (exposure time 0.5 s).

Figure 73. The serpentine channel configuration incorporated into flow-cell T.
These novel flow-cells were then compared to flow-cell A (conventional coiled-tubing approach). In general, the signals from the opaque chips were two-fold greater than those from flow-cell A (Figure 74). The response from flow-cells T and U were on average 7.8% and 2.8% greater than flow-cell S. The difference was greater at lower concentrations; at a morphine concentration of $1 \times 10^{-9}$ M, the signals from flow-cells T and U were 20.2% and 8.1% greater, respectively.

![Bar chart showing chemiluminescence intensity](image)

**Figure 74.** The effect of channel design on chemiluminescence intensity (peak height). Data for the reaction of $1 \times 10^{-7}$ M morphine with permanganate shown.

A visual examination of the light emitted from each flow-cell using the reaction of luminol with hydrogen peroxide and potassium hexacyanoferrate(III) revealed significant differences in solution mixing. In each case, this relatively long-lived reaction did not reach its maximum intensity until the solution had travelled a short distance in the reaction channel (Figure 75). In flow-cell S (spiral), the increase in intensity during the initial mixing period was most obvious near the walls of the channel (Figure 75i). This was attributed to the slower movement of solution in those...
regions (compared to the centre of the channel), which allowed greater reaction time over the same distance. The configuration of flow-cell U appeared to disrupt the gradual onset of light from the sides of the channel and reduced the distance required to reach the maximum intensity (Figure 75iii). However, the distribution of light during the initiation period suggested a slower movement of solution in the crests and troughs of the sinusoidal design (Figure 75iv). A more rapid onset of the emission was also observed in flow-cell T (serpentine), but the emission was more evenly distributed across the channel (Figure 75ii).

![Figure 75. Chemiluminescence from the reaction between luminol, potassium hexacyanoferrate(III) and hydrogen peroxide in the opaque (Acetal) flow-cells (i) S, (ii) T, (iii) U and (iv) zoomed in image of the initial turns of flow-cell U (contrast adjusted and converted to greyscale (0.3 s exposure time).](image)

The sudden changes in the direction of flow within flow-cell U, and to a greater extent within flow-cell T, create disturbances in a similar manner to knitted open tubular reactors [51] used for on-line derivatisation and photochemistry. This results in increased radial mixing and decreased axial dispersion, which in this case leads to greater chemiluminescence intensity. The effect of the channel configuration on
mixing efficiency was evident in photographs of the chemiluminescence from the reaction between the permanganate reagent and morphine, using an extended exposure time of 25 seconds (Figure 76).

In flow-cell S, the most intense emission occurred within the detection zone, but a significant amount of light was emitted as the solution passed along the exit channel (Figure 76i). Whilst in flow-cell U, the emission in the detection zone was more intense, and only a faint emission was observed in the exit line (Figure 76iii). In flow-cell T, the exit line was barely visible, even when using a longer exposure time of 90 seconds, confirming that under these conditions the chemiluminescence reaction was essentially complete before the solution exited the detection zone (Figure 76ii).

![Figure 76](image)

**Figure 76.** Chemiluminescence from the reaction between the standard permanganate reagent and morphine ($1 \times 10^{-3}$ M) in the opaque (Acetal) flow-cells (i) S, (ii) T and (iii) U (25 s exposure time).

### 3.4 Flow-cell comparison

As detailed in chapter 2, studies by our research group and others have shown:

(i) Repeated changes in the direction of solution flow (such as reversing turns) enhances the mixing efficiency and therefore the emission intensity [73, 81, 160, 231]. (ii) Relatively large chambers or wells in the detection zone generally delay the onset of the maximum emission as the solution rapidly moves through the path of least resistance, leaving surrounding areas of poor solution mixing [73, 188].
(iii) Channels machined or etched into an opaque white polymer and sealed with a transparent seal minimise the loss of light through surfaces not exposed to the photodetector [160, 169, 170]. (iv) A mixing tube/channel prior to the entrance of the detection zone is normally required for the reacting mixture to reach maximum emission intensity in front of the photodetector [71, 160, 231], but in the case of very rapid chemiluminescence systems, merging the solutions within the detection zone may be superior [188].

A variety of flow-cells that incorporate one or more of these advances were compared to the best opaque flow-cell (T) and the conventional coiled-tubing approach (A) to determine the effectiveness of each on the generation and transmission of light to the photomultiplier tube.

### 3.4.1 Flow injection analysis comparison

Ten flow-cell configurations were initially compared using the chemiluminescence reactions of morphine and amoxicillin with two acidic potassium permanganate reagents. As previously mentioned, the reaction of morphine with permanganate has been used extensively in both flow analysis and HPLC applications [25, 26]. Amoxicillin is a β-lactam antibiotic that has been detected with various permanganate chemiluminescence reagent systems [232-234]. Previous investigations involving these and other analytes (detailed in chapter 3) have shown that considerable increases in reaction rate and chemiluminescence intensity can be obtained by a preliminary partial reduction of permanganate to create a stable, relatively high concentration of manganese(III) in the reagent solution [205, 213]. The flow-cells were therefore also compared with the enhanced permanganate reactions as examples of extremely rapid chemiluminescence systems.
Using FIA methodology, the analytes were injected (10 replicates) into a water carrier stream, which merged with the permanganate reagent at a flow rate of 3.5 mL/min. In agreement with previous work [213], the enhanced reagent provided a considerable increase in chemiluminescence intensity (4-5 fold for morphine and 32-38 fold for amoxicillin for most flow-cells; Table 10). Greater enhancement (7-fold for morphine and 50-58 fold for amoxicillin) was observed for flow-cells F and H (dual-inlet serpentine machined into Teflon and Teflon impregnated with glass microspheres, respectively), where the merging of reactants within the detection zone enabled a greater proportion of the light emitted from these reactions to be captured (Figures 77 and 78).

<table>
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<td>33.0</td>
</tr>
<tr>
<td>W</td>
<td>5.2</td>
<td>37.6</td>
</tr>
<tr>
<td>X</td>
<td>4.2</td>
<td>37.8</td>
</tr>
<tr>
<td>Y</td>
<td>4.6</td>
<td>35.6</td>
</tr>
</tbody>
</table>

Several flow-cells provided greater chemiluminescence signals (Figure 77) than the traditional coiled-tubing approach (flow-cell A). Flow-cell W enabled the tubing to be tightly coiled on a reflective backing plate (Figure 79), the advantage of which
was most evident using the enhanced permanganate reactions. The GloCel detector (flow-cells C, D, G and H) provided a relatively inexpensive commercial option with a purpose-built housing. Machining the reaction channels into disks constructed from Teflon impregnated with 25% glass microspheres (flow-cells G and H) increased the signal intensities by up to 23%.

Although flow-cell T was also constructed by machining channels into a white polymer material, it produced significantly larger chemiluminescence signals (between 36% and 210% greater than the signals for flow-cells C, D, G and H; Figures 77 and 78). These differences can be attributed to the extremely thin transparent seal and the ability to place the photomultiplier tube flush against the chip, which enables a greater transfer of light to the photodetector.

![Figure 77. Comparison of chemiluminescence detection flow-cells for the oxidation of morphine (1 × 10⁻⁷ M) with the standard permanganate reagent (blue columns) and the enhanced permanganate reagent (pink columns).]
**Figure 78.** Comparison of chemiluminescence detection flow-cells for the oxidation of amoxicillin ($1 \times 10^{-5}$ M) with the standard permanganate reagent (blue columns) and the enhanced permanganate reagent (pink columns).

**Figure 79.** Chemiluminescence from the reaction between morphine ($1 \times 10^{-3}$ M) and the enhanced permanganate reagent in flow-cells (i) A, (ii) H, (iii) T and (iv) W. A 20 s exposure time was used for each photograph.
In agreement with work presented earlier in this chapter [160], flow-cells U and X, which were both constructed from transparent materials and backed with a mirror, produced much lower signals due to the loss of light through surfaces not exposed to the photodetector (Figure 80). The addition of a layer of aluminium foil around the surfaces of flow-cell Y enhanced the transmission of light to the detector by 25-44%. However, the absolute signals were still lower than most of the other flow-cells.

![Figure 80. Chemiluminescence from the reaction between morphine \((1 \times 10^{-5} \text{ M})\) and the enhanced permanganate reagent in flow-cells (i) U and (ii) X. A 30 s exposure time was used for each photograph.](image)

The greater chemiluminescence intensity obtained using flow-cell T also translated to a slightly lower limit of detection for amoxicillin \((9 \times 10^{-6} \text{ M})\) compared to the conventional coiled-tubing approach \((2 \times 10^{-7} \text{ M})\). Using the enhanced reagent, the detection limit was further extended to \(3 \times 10^{-9} \text{ M}\), which is superior to all previously reported values for this analyte with permanganate chemiluminescence [232, 233, 235].

### 3.4.2 High performance liquid chromatography

The flow-cells that provided the greatest chemiluminescence intensities with the enhanced permanganate system (H and T) were evaluated for post-column HPLC detection, in comparison with the conventional coiled-tubing approach (A). An initial comparison of these flow-cells was undertaken for the detection of a mixture of
α-adrenergic agonists from *C. aurantium* (Figure 81), due to the recent interest in their potentially adverse health effects as ingredients in weight-loss supplements [236]. Work previously conducted by Slezak et al. [21] and Percy et al. [22] has demonstrated the rapid determination of these analytes with monolithic column chromatography and acidic permanganate chemiluminescence detection. More recently, it has been shown that the emission intensities with these analytes can be improved by up to two-orders of magnitude with enhanced permanganate reagents, largely due to increased reaction rates that were more compatible with HPLC-based detection [205].

![Chemical Structures](image)

**Figure 81.** Structures of the α-adrenergic agonists from *C. aurantium*.

In this study a reverse-phase particle-packed column designed for highly aqueous mobile phases (98% Solvent A: deionised water adjusted to pH 2.15 with trifluoroacetic acid; 2% Solvent B: methanol) was employed. Separation times with this column were much longer than those in previous reports, providing greater peak resolution [21, 22].

Using flow-cell A, the enhanced reagent provided 15- to 49-fold larger signals than the standard reagent (Figure 82). The use of flow-cells H and T, which both contained channels machined into white polymer materials, further increased signal intensities by 10% and 55% respectively.
**Figure 82.** HPLC separation of *C. aurantium* compounds. Peaks: octopamine (OCT), synephrine (SYN), tyramine (TYR), N-methyltyramine (N-MET) and hordenine (HOR). Detection conditions: flow-cell A using the standard permanganate reagent (black trace); and flow-cells A (blue trace), H (pink trace) and T (yellow trace) using the enhanced permanganate reagent.

The flow-cells were also examined for the separation and detection of β-adrenergic agonist pharmaceuticals (Figure 83), which required higher proportions of organic modifier in the chromatographic mobile phase (up to 50% solvent B: methanol). Poor compatibility between the epoxy resin (used to attach the transparent seal to flow-cell T) and the HPLC mobile phase meant that it was not suitable for use in this analysis.
As only fenoterol had previously been detected with the enhanced reagent [213], the concentrations of permanganate and thiosulfate required to generate the greatest chemiluminescence intensities with each analyte were optimised using FIA (Figure 84), prior to implementing this detection system on the HPLC. Whilst the degree of enhancement was somewhat analyte dependant, the greatest intensities were generally obtained using a reagent containing 1.9 mM permanganate and 1.0 mM thiosulfate.
Figure 84. Optimisation of thiosulphate concentrations in reagents containing 1.0 mM permanganate (blue points) or 1.9 mM permanganate (pink points), using FIA for (i) epinephrine, (ii) isoprenaline, (iii) metaproterenol, (iv) salbutamol and (v) fenoterol.
The use of the enhanced reagent resulted in significant increases in chemiluminescence intensity for both salbutamol and fenoterol (Table 11). Moderate enhancement was obtained for metaproterenol (11-fold), and as a consequence, the extremely low response obtained with the standard reagent became comparable with that of epinephrine and isoprenaline. Similar trends were observed in the HPLC experiments (Figure 85 and Table 13), with discrepancies between the two experimental approaches attributable to differences in flow rates and the proportion of organic modifier in the carrier/eluent stream.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Chemiluminescence signal (mV)</th>
<th>Enhancement Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permanganate</td>
<td>Enhanced Permanganate</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>16.5</td>
<td>55.2</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>22.0</td>
<td>62.5</td>
</tr>
<tr>
<td>Metaproterenol</td>
<td>5.0</td>
<td>56.5</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>16.6</td>
<td>326.6</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>12.2</td>
<td>394.2</td>
</tr>
</tbody>
</table>

The limits of detection for the five analytes using FIA under these conditions (with flow-cell H) ranged from $1 \times 10^{-10}$ M to $5 \times 10^{-9}$ M (compared with $3 \times 10^{-8}$ M to $1 \times 10^{-7}$ M using the standard permanganate reagent, Table 12), which were better than all previously reported values for epinephrine, isoprenaline, salbutamol and fenoterol using permanganate chemiluminescence systems [213, 237-241]. Metaproterenol had not previously been detected with this reagent.
Table 12. Limits of detection for the $\beta$-adrenergic agonists using FIA methodology.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Standard permanganate</th>
<th>Enhanced permanganate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>$4.7 \times 10^{-4}$</td>
<td>$4.1 \times 10^{-6}$</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>$4.4 \times 10^{-4}$</td>
<td>$5.5 \times 10^{-6}$</td>
</tr>
<tr>
<td>Metaproterenol</td>
<td>$1.5 \times 10^{-7}$</td>
<td>$5.0 \times 10^{-9}$</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>$3.2 \times 10^{-8}$</td>
<td>$3.4 \times 10^{-10}$</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>$2.6 \times 10^{-8}$</td>
<td>$1.3 \times 10^{-10}$</td>
</tr>
</tbody>
</table>

*Calculated as $3 \times$ the standard deviation of the blank response.

The use of flow-cell F (dual-inlet serpentine channel machined into a Teflon disk) for post-column chemiluminescence detection provided an increase in signal of 34-69% across the five analytes, compared to flow-cell A with the same reagent (Figure 85). Flow-cell H, in which the Teflon disk was impregnated with glass microspheres, was on average 45% and 129% superior to flow-cells F and A, respectively.

Table 13. Relative chemiluminescence responses (peak areas) for the $\beta$-adrenergic agonists with the two permanganate reagents, using HPLC.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Chemiluminescence signal (mV)</th>
<th>Enhancement Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard permanganate</td>
<td>Enhanced permanganate</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>16.5</td>
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<tr>
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</tr>
<tr>
<td>Salbutamol</td>
<td>16.6</td>
<td>326.6</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>12.2</td>
<td>394.2</td>
</tr>
</tbody>
</table>
Figure 85. HPLC separation of β-adrenergic agonist pharmaceuticals. Peaks: epinephrine (EPI), isoprenaline (ISO), metaproterenol (MET), salbutamol (SAL) and fenoterol (FEN). Detection conditions: flow-cell A using the standard permanganate reagent (black trace); and flow-cells A (blue trace), D (pink trace) and H (yellow trace) using the enhanced permanganate reagent.
4. Conclusions

The construction of chemiluminescence detection flow-cells based on a coil of polymer tubing is an effective approach that has been used in both FIA and HPLC over decades of research. In flow-cell W, the signals from this simple design have been improved by the use of a reflective backing plate with grooves to ensure a tight coil of the transparent PTFE-PFA tubing.

Advances in computer-aided design and precision milling technology driven by microfluidics research can be exploited for the manufacture of flow-through cells for highly sensitive chemiluminescence detection in flow analysis. This approach allows exploration of channel configurations not attainable using conventional coiled-tubing flow-cells, to create superior designs that are more suitable for commercial production or scaling down for incorporation in microfluidic devices. Previously reported chemiluminescence reactors constructed by machining channels in polymers (for flow analysis or microfluidic applications) have almost exclusively been prepared using transparent materials, but far greater emission intensities can be obtained using opaque polymers with a transparent seal. Further increases in the emission intensity from these relatively fast chemiluminescence reactions can be achieved by incorporating channels that produce sudden changes in the direction of flow to enhance mixing efficiency.

Comparison of several novel flow-cells revealed the importance of surface reflectance. The introduction of glass microspheres in the Teflon disks of the GloCel detector (flow-cells G and H) improved the transmission of light to the photodetector. Even greater signals were observed from an Acetal chip with a similar channel configuration (flow-cell D), which may in part arise due to the shorter distance from the reaction channel to the photomultiplier window. Despite
incorporating highly reflective back surfaces, the two flow-cells constructed from transparent materials (polycarbonate and glass) produced inferior signals due to the loss of light from the sides of the chips.

A further advantage of machining into polymer materials is the inclusion of threaded inlet/outlet ports for standard fittings (which overcomes problems associated with pressure at high flow rates) and custom designs for incorporation in purpose-built light-tight housings. The combination of these flow-cells, which provide efficient mixing of fast chemiluminescence reactions close to or at the point of detection, with the enhanced permanganate reagent has improved limits of detection for a range of organic analytes. The findings of this chapter have resulted in two recent publications [160, 242].
Determination of *Papaver somniferum* alkaloids in drug seizure samples using high performance liquid chromatography with UV-absorbance and chemiluminescence detection

- Introduction
- Experimental
- Results and discussion
- Conclusion
1. Introduction

Heroin (3,6-diacetylmorphine) is a semi-synthetic compound derived from the acetylation of the *Papaver somniferum* opiate alkaloid morphine [125, 133-135]. Originally synthesised as a non-addictive morphine substitute, heroin was introduced into the commercial market as a cough suppressant and treatment for tuberculosis [125, 133-135]. However, as addiction to these medications rapidly increased, it was discovered that by simply acetylating the phenolic and hydroxyl moieties (on carbons 3 and 6 respectively of the phenanthrene backbone), a narcotic even more powerful and addictive than its parent compound had been created [125, 133-135]. The ongoing illicit production and trafficking of heroin has resulted in it being considered as one of the most significantly abused narcotics [128, 129]. This has created the need to identify and/or quantify the drug (and its precursors or metabolites) in suspected drug seizure samples.

Screening tests are often utilised to obtain rapid preliminary identification of certain drugs or classes of drugs prior to confirmatory testing with more specific instrumental methods [125, 128, 129, 145, 146]. The most commonly used screening method for opiates and opiate derivatives involves mixing seizure samples with reagents such as Mandelin’s, Marquis or Mecke’s and monitoring the resultant colour change [125, 128, 129, 145, 146]. These spot tests are usually selective for a particular class of drug (i.e. opiates), but are not selective for specific drugs (i.e. heroin), so a series of tests is usually performed [125, 128, 129, 145, 146].

Microcrystalline tests have been used to provide greater specificity, however, the presence of cutting agents and diluents can result in significant alterations to the crystal structure which can impede identification [125, 128, 129, 145].
As previously mentioned, chemiluminescence is a highly selective and sensitive method of detection that has been applied to various flow based techniques [1-3, 6-8]. Our research group has demonstrated the analytical utility of chemiluminescence reactions, in particular those of acidic potassium permanganate and tris(2,2′-bipyridine)ruthenium(III) for the determination of various opiate alkaloids in process samples using FIA, SIA, HPLC and CE [148-151, 153, 154, 156, 243]. The complimentary selectivity of these reagents for phenolic and non-phenolic opiates served as the basis of a rapid screening test for heroin developed by Agg and co-workers [27, 158].

In stage one of the screening test, a suspected heroin seizure sample is reacted with the tris(2,2′-bipyridine)ruthenium(III) reagent [27, 158]. The production of a strong chemiluminescence response is suggestive that the sample may contain heroin [27, 158]. Preliminary confirmation occurs in stage two of the test which involves mixing the seizure sample with concentrated base prior to reaction with acidic potassium permanganate [27, 158]. If the sample contains heroin it will be rapidly hydrolysed to morphine and 6-monoacetylmorphine, which produce intense chemiluminescence responses with the second reagent [27, 158].

Some tertiary amines (such as codeine, strychnine and chloroquine) have been found to elicit false positives with the tris(2,2′-bipyridine)ruthenium(III) reagent [27, 158]. However, following hydrolysis, these species fail to elicit an emission with acidic potassium permanganate to rival that of morphine and 6-monoacetylmorphine [27, 158]. This rapid screening test has been successfully employed for the determination of heroin in seizure samples using both FIA and SIA methodologies [27, 158]. However, an inherent disadvantage of performing this test with these techniques is the lack of a separative step, which has made it almost impossible to
identify the presence of multiple opiates and/or opiate impurities within these complex matrices.

The use of HPLC coupled with chemiluminescence detection has previously been shown to be an effective means of monitoring opiate alkaloids in process samples [25, 26, 153, 154, 243, 244]. However, this methodology has not yet been utilised for the rapid screening test for heroin.

Camenzuli and co-workers [245, 246] have recently designed a chromatographic column equipped with an annular frit to enable processing of flow streams in a manner that emulates conventional post-column detection [245, 246]. The frit is contained in an outlet fitting equipped with multiple exit ports: a central port to capture flow eluting from the central section of the column bed, and three exit ports that enable the peripheral portion of flow to be captured [245, 246]. This column technology has the potential to allow multiple post-column detectors (i.e. UV-absorbance, chemiluminescence, fluorescence, mass spectrometry etc) to be coupled for simultaneous analysis in a way that until now, has not been feasible using conventional approaches.

In recent years, there have been several major advances in two of the most prolifically used chemiluminescence reagent systems (acidic potassium permanganate and tris(2,2'-bipyridine)ruthenium(III)), in terms of their stability [33, 205, 213] and sensitivity [205, 213], and the understanding of their light producing pathways [14, 216, 217]. As such, this chapter details the implementation of a rapid HPLC based post-column chemiluminescence screening test for opiate alkaloids and opiate derivatives in drug seizure samples using the more stable and sensitive forms of the acidic potassium permanganate and tris(2,2'-bipyridine)ruthenium(III)
reagents. The novel column technology described above was utilised to simultaneously determine these opiates with three unique modes of detection.
2. Experimental

2.1 Chemicals and reagents

Deionised water (Continental Water Systems, Victoria, Australia) and analytical grade reagents were used unless otherwise stated. Chemicals were obtained from the following sources: sodium polyphosphate (+80 mesh) and trifluoroacetic acid from Sigma-Aldrich (New South Wales, Australia); sodium thiosulfate from Fluka, (New South Wales, Australia); potassium permanganate from Chem-Supply (South Australia, Australia); lead dioxide, sodium hydroxide and sodium perchlorate from Ajax Finechem (New South Wales, Australia); methanol and sulfuric acid from Merck (Victoria, Australia); glacial acetic acid and perchloric acid (70% w/v) from Univar (New South Wales, Australia); acetonitrile from Burdick & Jackson (Michigan, USA) and tris(2,2'-bipyridine) ruthenium(II) dichloride hexahydrate from Strem Chemicals (Minnesota, USA). Codeine, heroin (≥85%), morphine, oripavine, papaverine and thebaine were provided by GlaxoSmithKline (Victoria, Australia). Drug seizure samples were provided by the Australian Federal Police.

The ‘enhanced’ permanganate reagent was prepared by dissolution of potassium permanganate (1.9 \times 10^{-2} \text{ M}) in 1% (m/v) sodium polyphosphate and adjusting to pH 2.5 with sulfuric acid and then adding sodium thiosulfate (0.6 mM), using a small volume of a 0.1 M solution.

The ‘stabilised’ tris(2,2′-bipyridine) ruthenium(III) reagent was prepared as previously described [33]. Briefly, this involved treating [Ru(bipy)_3]Cl_2 with sodium perchlorate in aqueous solution to yield the characteristic bright orange [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 hours. The reagent
solution was prepared by oxidising the \([\text{Ru(bipy)}_3]^{2+}(\text{ClO}_4)_2\) crystals \((1 \times 10^{-3} \text{ M})\) with lead dioxide \((0.2 \text{ g/100 mL})\) in acetonitrile containing 0.05 M perchloric acid. Following 1 minute of vigorous mixing and the characteristic change in colour from orange to blue-green, the resultant suspension was allowed to settle prior to filtration through an in-line filter (consisting of a small Pasteur pipette packed tightly with glass wool).

Stock solutions of the opiate alkaloids (morphine, oripavine, codeine, thebaine and papaverine, \(1 \times 10^{-3} \text{ M}\)) were prepared by dissolution in acidified deionised water. Heroin \((1 \times 10^{-3} \text{ M})\) was prepared in 0.1% (v/v) acetic acid and diluted in 0.05% (v/v) acetic acid.

Stock solutions of the drug seizure samples were prepared by dissolving approximately 15 mg of the solid material into 50 mL of 0.1% (v/v) acetic acid.

The ‘non-hydrolysed’ samples were prepared by taking 1.0 mL of stock solution (heroin or the drug seizure sample) and diluting to either 100 mL (HPLC with multiplexed detection) or 200 mL (FIA/ HPLC with conventional end column detection) with a 0.05% (v/v) acetic acid solution.

The ‘hydrolysed’ samples were prepared by mixing 1.0 mL of stock solution with 100 \(\mu\text{L}\) of sodium hydroxide \((1.0 \text{ M})\) and then diluting the mixture to 100 mL or 200 mL with the 0.05% (v/v) acetic acid solution.

### 2.2 Flow injection analysis

The FIA manifold was constructed from a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Victoria, Australia) with bridged PVC pump tubing \((1.02 \text{ mm i.d.; DKSH, Queensland, Australia})\), black manifold tubing \((0.76 \text{ mm i.d.,})\),
Global FIA, Washington, USA), six-port injection valve (Vici 04 W-0192L, Valco Instruments, Texas, USA) and an in-house fabricated detector containing flow-cell W (a tight coil of Teflon tubing attached to an aluminium back-plate) mounted flush against the window of an extended range photomultiplier module (Electron Tubes P30A-05, ETP, New South Wales, Australia) within light-tight housing. All tubing entering and exiting the detector was black PTFE (0.76 mm i.d., Global FIA). The output signal from the detector was recorded with an ‘e-corder 410’ data acquisition system (eDAQ, New South Wales, Australia).

The analytes ($5 \times 10^{-6}$ M) were injected (70 µL) into a 100% aqueous carrier stream (deionised water adjusted to pH 2.5 with trifluoroacetic acid, 3.5 mL/min) that merged with a flowing stream of the chemiluminescence reagent(s) (3.5 mL/min) at a confluence point located immediately prior to the inlet of the flow-cell (Figure 86).

![Figure 86. Schematic of the FIA manifold.](image)

### 2.3 High performance liquid chromatography

Analyses were carried out on an Agilent Technologies 1200 series liquid chromatography system, equipped with a quaternary pump, solvent degasser system and autosampler (Agilent Technologies, Victoria, Australia), using a reversed phase Hypersil Gold chromatography column (100 mm × 4.6 mm i.d., 5 µm, ThermoFisher...
Scientific, Cheshire, United Kingdom), with an injection volume of 20 µL, flow rate of 2.5 mL/min and a UV-absorbance detector operating at 280 nm (Agilent Technologies). An analogue 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.

Opiate standards were separated via gradient elution with deionised water adjusted to pH 2.5 with trifluoroacetic acid (solvent A) and methanol (solvent B) as follows: 0-1 min: 5-10% B, 1-2 min: 10-25% B, 2-6 min: 25-35% B, 6-6.5 min: 35% B, 6.5-8 min: 35-5% B, 8-9 min: 5% B.

2.3.1 Conventional high performance liquid chromatography with post-column chemiluminescence detection

The column eluate (2.5 mL/min) and chemiluminescence reagent(s) (1.0 mL/min) merged at a confluence point located immediately prior to or within the detection zone of the flow-cells (Figure 87). A GloCel detector (Global FIA, Washington, USA) equipped with flow-cell D and extended range photomultiplier module (Electron Tubes, model P30A-05, ETP, New South Wales, Australia) was used for the permanganate experiments, whilst an in-house fabricated detector consisting of flow-cell W and an extended range photomultiplier module within light-tight housing was used for the tris(2,2'-bipyridine)ruthenium(III) experiments.
2.3.2  High performance liquid chromatography with multiplexed detection

The column eluate (2.5 mL/min) was segmented through the annular frit in such a way that (i) 27.4% (flow rate 0.68 mL/min) exited peripheral port 1 and entered the UV-absorbance detector, (ii) 30.4% (flow rate 0.76 mL/min) exited peripheral port 2 and merged with the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent (1.0 mL/min) at a confluence point located immediately prior to flow-cell W (as described in section 2.3.1), (iii) 29.8% (flow rate 0.74 mL/min) exited peripheral port 3 and merged with the enhanced permanganate reagent (1.0 mL/min) at a confluence point located within the GloCel detector (Global FIA, Washington, USA) equipped with flow-cell D (as described in section 2.3.2) and (iv) 12.4% (flow rate 0.31 mL/min) exited the central port to waste (Figure 88).
Where:

(i) UV-absorbance detection

(ii) Stabilised tris(2,2′-bipyridine)ruthenium(III) chemiluminescence reagent

(iii) Enhanced permanganate chemiluminescence reagent

(iv) Waste

Figure 88. Schematic of the HPLC manifold employed for multiplexed UV-absorbance and chemiluminescence detection.
3. Results and discussion

3.1 Initial experiments

Preliminary FIA experiments were conducted to examine the responses from a pure heroin standard solution, prepared with and without the hydrolysis step, upon reaction with the two chemiluminescence reagents. In accordance with the observations of Agg et al. [158], a large response was obtained from the reaction of heroin with the stabilised tris(2,2′-bipyridine)ruthenium(III) reagent and a negligible response with the enhanced permanganate reagent (Figure 89). Following hydrolysis of the sample, a modest response was observed with the stabilised tris(2,2′-bipyridine)ruthenium(III) reagent and an intense response observed with enhanced permanganate.

![Chemiluminescence reagent](image)

**Figure 89.** Comparison of the relative chemiluminescence responses (peak heights) for a non-hydrolysed (blue columns) and hydrolysed (pink columns) heroin standard ($5 \times 10^{-6}$ M) with the stabilised tris(2,2′-bipyridine)ruthenium(III) and enhanced permanganate reagents. The chemiluminescence responses have been normalised for each reagent.
3.1.1 Application to real samples

Six drug seizure samples were obtained from the Australian Federal Police and examined using FIA methodology. The relative signal intensities (peak heights) with the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent (Figure 90) and the enhanced permanganate reagent (Figure 91) for the non-hydrolysed and hydrolysed samples are shown below.

The chemiluminescence signals for the hydrolysed samples upon reaction with the tris(2,2'-bipyridine)ruthenium(III) reagent were between 66% and 81% of that observed from the corresponding non-hydrolysed samples (Figure 90). This is attributable to the conversion of heroin into two phenolic morphinan alkaloids, 6-monoacetylmorphine and morphine, which quench the response of the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent. The intense signals obtained from the hydrolysed seizure samples may signify the presence of naturally occurring non-phenolic opiate impurities such as 6-acetylmorphine, which is often present in opium extracts [128, 129].

When reacted with the enhanced permanganate reagent, the non-hydrolysed samples produced moderate responses (Figure 91). However, following hydrolysis, a significant increase in chemiluminescence intensity was observed. Whilst the absolute chemiluminescence intensities varied, four of the non-hydrolysed seizure samples produced responses between 3% and 11% of the corresponding hydrolysed sample. Non-hydrolysed samples 1 and 6 produced responses that were 26% and 63% of their corresponding hydrolysed sample. This may be attributable to the presence of significant quantities of 6-monoacetylmorphine formed via natural degradation of the samples following synthesis.
Figure 90. Chemiluminescence responses (peak heights) for the non-hydrolysed (blue columns) and hydrolysed (pink columns) heroin seizure samples with the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent.

Figure 91. Chemiluminescence responses (peak heights) for the non-hydrolysed (blue columns) and hydrolysed (pink columns) heroin seizure samples with the enhanced permanganate reagent.
These results demonstrate that the combination of two chemiluminescence reagents, coupled with a simple hydrolysis step, provide an effective method of screening for heroin in drug seizure samples. However, an inherent disadvantage of performing this test with FIA methodology is the lack of a separative step, which has, to-date, prevented the identification of multiple opiates and/or opiate impurities within these samples.

3.2 Conventional high performance liquid chromatography with post-column UV-absorbance detection

Camenzuli and co-workers [245, 246] have recently designed a chromatographic column equipped with an annular frit that enabled processing of flow streams in a manner that emulated conventional post-column detection. The capacity to simultaneously employ multiple modes of detection should enable reductions in (i) total analysis time, (ii) sample and/or solvent consumption and (iii) waste generation for various analytical methodologies, including the rapid screening test for heroin.

The performance of this column was initially evaluated by sealing each of the peripheral exit ports of the annular frit, allowing passage of the solvent through only the central outlet (emulating conventional post-column detection, Figure 92).

![Figure 92. Illustration of the column outlet showing the three peripheral exit ports and the single central exit port.](image-url)
3.2.1  *Optimisation of separation conditions*

Initial separation experiments were conducted with UV-absorbance detection. An optimisation of HPLC conditions (mobile phase composition, solvent gradient and flow rate) was undertaken to rapidly separate heroin from several *P. somniferum* opiate alkaloids (morphine, codeine, oripavine, thebaine, and papaverine, Figure 93).

![Chemical structures](image)

*Figure 93. Structures of the *P. somniferum* opiate alkaloids.*

The compatibility between the chemical parameters necessary for an efficient chromatographic separation and sensitive chemiluminescence detection is extremely important [5, 6, 9, 52]. Both acetonitrile and methanol have been used as an organic modifier in HPLC procedures employing post-column tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection [30, 31]. However, several studies have shown that incorporating acetonitrile into the mobile phase resulted in significant reductions in signal intensity or complete quenching of the emission with acidic potassium permanganate reagents [13, 75, 153, 154, 243, 247]. In contrast, the addition of methanol causes negligible changes to the background
response and signal intensity [13, 75, 153, 154, 243, 247]. As the column eluate was to be segmented between detectors employing tris(2,2′-bipyridine)ruthenium(III) and permanganate based chemiluminescence reagents, methanol was selected as the organic modifier for this separation.

The solvent gradient and flow rate through the column were optimised to afford a rapid separation that provided sufficient resolution between all analytes, whilst maintaining satisfactory pressure within the column (< 200 bar). The gradient conditions outlined by Agg and co-workers [158] were initially employed, however poor resolution between thebaine, heroin and papaverine resulted in a modification of the gradient to that described in the experimental. The flow rate was increased from 1.0 mL/min to 2.5 mL/min with no loss of resolution between the six opiate alkaloids, and a significant reduction in run time (from 11.5 minutes to 6.5 minutes; Figure 94).

![Figure 94. HPLC separation of a mixture of *P. somniferum* opiate alkaloids (5 x 10^{-6} M) with UV-absorbance detection at 280 nm. Peaks: morphine (MOR), codeine (COD), oripavine (ORI), thebaine (THE), heroin (HER) and papaverine (PAP).](image_url)
To establish the sensitivity of UV-absorbance detection, calibration curves were prepared for each analyte using seventeen standard solutions between $1 \times 10^{-9}$ M and $1 \times 10^{-5}$ M. As demonstrated by the correlation co-efficients ($R^2$, Table 14), each of the opiates exhibited a highly linear relationship between absorbance (peak area) and analyte concentration. The detection limits, defined as a signal to noise ratio of 3 were in the range of $5 \times 10^{-8}$ M and $5 \times 10^{-7}$ M. The precision of repeated injections was adequate, with relative standard deviations between 2.96% and 4.37%.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Calibration function (peak area, arbitrary units)</th>
<th>$R^2$</th>
<th>LOD (M)$^a$</th>
<th>R.S.D. %$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>$y = 6.29 \times 10^7 x + 0.001$</td>
<td>0.9986</td>
<td>$2.5 \times 10^{-7}$</td>
<td>2.96</td>
</tr>
<tr>
<td>Codeine</td>
<td>$y = 6.89 \times 10^7 x - 0.161$</td>
<td>0.9980</td>
<td>$5.0 \times 10^{-7}$</td>
<td>3.62</td>
</tr>
<tr>
<td>Oripavine</td>
<td>$y = 3.35 \times 10^6 x - 0.404$</td>
<td>0.9997</td>
<td>$1.0 \times 10^{-7}$</td>
<td>3.41</td>
</tr>
<tr>
<td>Thebaine</td>
<td>$y = 3.10 \times 10^6 x - 0.040$</td>
<td>0.9996</td>
<td>$5.0 \times 10^{-8}$</td>
<td>4.37</td>
</tr>
<tr>
<td>Heroin</td>
<td>$y = 7.85 \times 10^5 x + 0.303$</td>
<td>0.9992</td>
<td>$5.0 \times 10^{-8}$</td>
<td>3.13</td>
</tr>
<tr>
<td>Papaverine</td>
<td>$y = 3.80 \times 10^6 x - 0.301$</td>
<td>0.9997</td>
<td>$1.0 \times 10^{-7}$</td>
<td>3.21</td>
</tr>
</tbody>
</table>

$^a$The lowest response detected with a signal to noise ratio of 3.
$^*$n = 5; $1 \times 10^{-5}$ M

### 3.2.2 Application to real samples

The aforementioned separation conditions were utilised to determine the heroin content of six drug seizure samples, each of which were within ranges previously reported by other researchers [248-254] (Table 15).
Table 15. Concentration of heroin in drug seizure samples obtained with UV-absorbance detection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heroin (mass percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
</tr>
</tbody>
</table>

Whilst the absolute signal intensities varied, heroin (retention time 4.7 minutes) was detected in each of the non-hydrolysed seizure samples (Figure 95). A response from an unknown compound was observed in five of the non-hydrolysed and hydrolysed samples (retention time at 3.0 minutes; Figure 95i, ii, iv, v and vi). This may be attributable to the presence of naturally occurring opiate impurities in the opium extract used to manufacture these samples. Elucidation of this species and its precursor would require further investigation.

Following hydrolysis, morphine (retention time 1.7 minutes) and/or 6-monoacetylmorphine (retention time 2.9 min, Figure 95) were detected in each of the samples, with the peak at 4.7 minutes (heroin) noticeably absent.
Figure 95. Chromatograms obtained for the analysis of six drug seizure samples with UV-absorbance detection at 280 nm. Pink and black traces show sample preparation with and without hydrolysis. Peaks: morphine (MOR), 6-monoaceoty morphine (6-MAM) and heroin (HER).
3.3 Conventional high performance liquid chromatography with post-column tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection

Greater selectivity and sensitivity can be derived from coupling HPLC with chemiluminescence detection [5, 6, 9, 52]. In recent years, the limited temporal stability of chemically oxidised tris(2,2'-bipyridine)ruthenium(III) in aqueous solutions has significantly impeded its use as a post-column chemiluminescence reagent [1, 5, 30, 31, 33-36]. To overcome this, McDermott and co-workers [33] recently synthesised [Ru(bipy)$_3$](ClO$_4$)$_2$, which (when dissolved in acetonitrile containing 0.05 M perchloric acid and oxidised with lead dioxide) was successfully applied as a post-column chemiluminescence reagent. This enabled analysis without the need for constant re-calibration or preparation of fresh reagent solutions over extended periods of time (48 hours) [33].

Using the optimised conditions (from section 3.2.1), a mixture of the six opiates (5 x 10$^{-6}$ M) was separated. Only codeine, thebaine and heroin (retention times of 2.6, 4.3 and 4.7 minutes respectively) elicited a response with the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent, demonstrating its selectivity for non-phenolic morphinan alkaloids (Figure 96).
**Figure 96.** HPLC separation of a mixture of *P. somniferum* opiate alkaloids (5 × 10⁻⁶ M) with stabilised tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection. Peaks: codeine (COD), thebaine (THE) and heroin (HER).

The effect of reagent flow rate on emission intensity was examined between 1.0 mL/min and 3.5 mL/min. A flow rate of 1.0 mL/min was found to afford the greatest chemiluminescence response for each of the three analytes and was selected for use in all further experiments.

Analytical figures of merit were obtained for codeine, thebaine and heroin (Table 16). Up to two-orders of magnitude greater sensitivity was achieved for the three non-phenolic morphinan alkaloids using the stabilised ruthenium chemiluminescence reagent (when compared to UV-absorbance). These limits of detection (between 1 × 10⁻⁹ M and 2.5 × 10⁻⁹ M) are comparable to, or better than, previously reported limits of detection for these analytes with tris(2,2'-bipyridine)ruthenium(III) chemiluminescence [35, 153, 154, 156, 157, 165]. Of further note were the improvements in linearity ($R^2$) and precision (% R.S.D.).
Table 16. Analytical figures of merit obtained with the stabilised ruthenium reagent.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Calibration function (peak area, arbitrary units)</th>
<th>R²</th>
<th>LOD (M)</th>
<th>R.S.D. %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>$y = 1.40 \times 10^8 x - 10.308$</td>
<td>0.9998</td>
<td>$1.0 \times 10^{-9}$</td>
<td>0.57</td>
</tr>
<tr>
<td>Thebaine</td>
<td>$y = 7.30 \times 10^5 x + 4.306$</td>
<td>0.9997</td>
<td>$2.5 \times 10^{-9}$</td>
<td>0.89</td>
</tr>
<tr>
<td>Heroin</td>
<td>$y = 2.40 \times 10^3 x + 8.181$</td>
<td>0.9972</td>
<td>$1.0 \times 10^{-9}$</td>
<td>0.74</td>
</tr>
</tbody>
</table>

*The lowest response detected with a signal to noise ratio of 3.

*n = 5; 1 × 10⁻⁶ M

3.3.1 Application to real samples

The improved selectivity and sensitivity of the stabilised tris(2,2'-bipyridine)ruthenium(III) reagent afforded much simpler chromatograms when applied to the post-column determination of the six drug seizure samples. Heroin was detected in each, with strong responses from samples 1, 3 and 5 (Figure 97i, iii and v), consistent with results obtained during the preliminary experiments conducted with FIA methodology. The chemiluminescence signals for the hydrolysed samples (upon reaction with the tris(2,2'-bipyridine)ruthenium(III) reagent) were between 38% and 60% of that observed from the corresponding non-hydrolysed samples (Figure 97). This is attributable to the conversion of heroin into 6-monoacetyl morphine and morphine, which quench the emission from the tris(2,2'-bipyridine)ruthenium(III) reagent [31, 40, 41]. A response from the unknown compound initially detected using UV-absorbance was also observed in each of the hydrolysed samples. This provides a clue with regard to elucidating its molecular structure, as the tris(2,2'-bipyridine)ruthenium(III) reagent is highly selective for non-phenolic opiates [30, 31, 41, 156]. However, as previously mentioned, further investigation is required to positively identify this species.
Figure 97. Chromatograms obtained for the analysis of six drug seizure samples with stabilised tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection. Pink and black traces show sample preparation with and without hydrolysis. Peaks: heroin (HER).
The heroin content of the six samples (Table 17) was found to be within the range previously reported [248-254] and correlated well with the results obtained using UV-absorbance detection. The difference between the two modes of detection ranged between 1.45% and 10.42% across the six samples.

Table 17. Concentration of heroin in drug seizure samples obtained with stabilised tris(2,2′-bipyridine)ruthenium(III) chemiluminescence detection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heroin (percent mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
</tr>
</tbody>
</table>

3.4 Conventional high performance liquid chromatography with post-column enhanced permanganate chemiluminescence detection

As previously discussed, the preliminary partial reduction of manganese(VII) by sodium thiosulfate creates a stable, relatively high concentration of manganese(III) in permanganate reagents which provides two major advantages for chemiluminescence detection; (i) improved temporal stability (a negligible change in peak area over 48 hours; < 1% based on the line of best fit) and (ii) dramatic increases in reaction rates translating to enhanced limits of detection for various analytes [213]. These features are highly compatible with the extended analysis periods utilised in many chromatographic procedures and may provide superior analytical figures of merit for the post-column detection of opiate alkaloids [213].

The enhanced permanganate reagent is highly selective for phenolic morphinan alkaloids [213], and as such when a mixture of the six opiates ($5 \times 10^{-6}$ M) was
separated, only morphine and oripavine (retention times of 1.7 and 2.85 minutes respectively) elicited a response (Figure 98). A small peak from an unknown compound was observed at 2.9 minutes. This was believed to be 6-monoacetylmorphine, present in the opiate mix as a result of minor degradation of the heroin standard. The identity of this peak was confirmed by analysing a 6-monoacetylmorphine standard \((5 \times 10^{-6} \text{ M})\) and obtaining an identical retention time. A small degree of co-elution occurred between the oripavine and 6-monoacetylmorphine peaks. This was deemed to be insignificant and due to the anticipated large increase in the 6-monoacetylmorphine peak following hydrolysis of the seizure samples, the separation conditions were not altered.

**Figure 98.** HPLC separation of a mixture of *P. somniferum* opiate alkaloids \((5 \times 10^{-6} \text{ M})\) with enhanced permanganate chemiluminescence detection. Peaks: morphine (MOR), oripavine (ORI) and 6-monoacetylmorphine (6-MAM).

The use of the enhanced permanganate reagent provided two-and-a-half-orders of magnitude greater sensitivity than UV-absorbance, with limits of detection for
morphine and oripavine of $7.5 \times 10^{10}$ M and $1 \times 10^{9}$ M respectively (Table 18). These are comparable to or better than previously reported limits of detection for these analytes with acidic potassium permanganate chemiluminescence [13, 75, 147, 148, 150, 153, 154, 243, 247].

Table 18. Analytical figures of merit obtained with enhanced permanganate chemiluminescence detection.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Calibration function (peak area, arbitrary units)</th>
<th>$R^2$</th>
<th>LOD (M)$^a$</th>
<th>R.S.D. (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>$y = 8.54 \times 10^2x + 15.827$</td>
<td>0.9993</td>
<td>$7.5 \times 10^{10}$</td>
<td>0.67</td>
</tr>
<tr>
<td>Oripavine</td>
<td>$y = 1.13 \times 10^2x + 2.657$</td>
<td>0.9999</td>
<td>$1.0 \times 10^{9}$</td>
<td>0.37</td>
</tr>
</tbody>
</table>

$^a$ The lowest response detected with a signal to noise ratio of 3.
$^\text{n} = 5; 1 \times 10^{-8}$ M

3.4.1 Application to real samples

When the non-hydrolysed drug seizure samples were screened with the enhanced permanganate reagent, 6-monoacetylmorphine and/or morphine were detected in each (Figure 99). Samples 1, 2, 4 and 6 generated particularly intense 6-monoacetylmorphine peaks, which is consistent with results obtained using FIA methodology. Following hydrolysis, a significant increase in chemiluminescence intensity was observed for both morphine and 6-monoacetylmorphine across the six seizure samples. Whilst the absolute chemiluminescence intensities varied, non-hydrolysed samples 3 and 5 produced responses between 3.8% and 6% of their corresponding hydrolysed samples. The remaining non-hydrolysed seizure samples produced responses for 6-monacetylmorphine that were between 29% and 41% of their corresponding hydrolysed samples. This may be attributable to the presence of significant quantities of 6-monoacetylmorphine formed via natural degradation of the samples following synthesis.
Figure 99. Chromatograms obtained for the analysis of six drug seizure samples with enhanced permanganate chemiluminescence detection. Pink and black traces show sample preparation with and without hydrolysis. Peaks: morphine (MOR) and 6-monacetylmorphine (6-MAM).
3.5 High performance liquid chromatography with multiplexed UV-absorbance and chemiluminescence detection

As previously mentioned, the column was equipped with an annular frit that enabled flow from the central and/or peripheral portions of the column bed to be segmented through multiple detectors. Therefore each port of the annular frit was attached to an individual detector or fed through to waste (Figure 100), enabling the simultaneous detection of opiate alkaloids in suspected heroin seizure samples with UV-absorbance and two chemiluminescence reagents.

![Diagram of column outlet](image)

**Figure 100.** Illustration of the column outlet showing the three detectors attached to the peripheral exit ports and the single central exit port leading to waste.

Using the separation conditions optimised for conventional post-column detection, a mixture of the six opiates \( (5 \times 10^{-6} \text{ M}) \) was separated and simultaneously determined by the three detectors. When the chromatograms from the two modes of UV detection (conventional and multiplex) were compared, it was discovered that the peaks were essentially coincident in time, but the multiplex approach appeared to have poorer sensitivity (Figure 101). By equipping each port of the annular frit with a detector (or line through to waste), the total flow from the column was segmented between the central and peripheral ports, resulting in much lower flow rates passing through the detectors than when using conventional post-column HPLC. Consequently the flow rates through the three detectors were on average 3.4-fold
slower than when using the conventional post-column approach (0.73 mL/min compared to 2.5 mL/min). A closer examination of these chromatograms revealed that whilst the absolute intensities (peak heights) were considerably lower than that of the conventional mode, the peaks were significantly broader (due to the longer residence time in the UV-absorbance flow-cell), and consequently the peak areas were comparable.

![Graph](image)

**Figure 101.** HPLC separation of a mixture of *P. somniferum* opiate alkaloids (2.5 × 10^−6 M) with conventional post-column UV-absorbance detection (black trace) and multiplexed UV-absorbance detection (pink trace). Peaks: morphine (MOR), codeine (COD), oripavine (ORI), thebaine (THE), heroin (HER) and papaverine (PAP).

Analytical figures of merit were obtained for the six opiate alkaloids in the three multiplexed detectors (Tables 19, 20 and 21). In general, the limits of detection obtained via the multiplexed approach were slightly inferior (less than an order of magnitude) to those achieved with conventional post-column detection. This reduction in sensitivity did not pose a problem for the rapid screening test, as the concentration of heroin in suspected seizure samples is typically much higher than
the limits of detection obtained using the multiplexed approach. The precision of repeated injections (% R.S.D.) was also somewhat poorer; however this is most likely attributable to sampling different regions of the peripheral portion of solution flow.

The ability to simultaneously run three modes of detection resulted in considerable reductions in sample and solvent consumption, waste generation as well as total analysis time (from 30 hours to 10 hours).

Table 19. Analytical figures of merit obtained with UV-absorbance detection.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Calibration function (peak area, arbitrary units)</th>
<th>$R^2$</th>
<th>LOD (M)$^a$</th>
<th>R.S.D. %$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>$y = 6.66 \times 10^5 x - 0.102$</td>
<td>0.9979</td>
<td>$7.5 \times 10^{-7}$</td>
<td>3.0</td>
</tr>
<tr>
<td>Codeine</td>
<td>$y = 5.43 \times 10^5 x - 0.151$</td>
<td>0.9998</td>
<td>$7.5 \times 10^{-7}$</td>
<td>3.3</td>
</tr>
<tr>
<td>Oripavine</td>
<td>$y = 3.49 \times 10^5 x - 1.786$</td>
<td>0.9984</td>
<td>$5.0 \times 10^{-7}$</td>
<td>3.9</td>
</tr>
<tr>
<td>Thebaine</td>
<td>$y = 3.03 \times 10^5 x - 0.004$</td>
<td>0.9992</td>
<td>$2.5 \times 10^{-7}$</td>
<td>2.8</td>
</tr>
<tr>
<td>Heroin</td>
<td>$y = 2.30 \times 10^5 x + 2.074$</td>
<td>0.9935</td>
<td>$5.0 \times 10^{-7}$</td>
<td>3.0</td>
</tr>
<tr>
<td>Papaverine</td>
<td>$y = 2.39 \times 10^5 x - 0.187$</td>
<td>0.9987</td>
<td>$2.5 \times 10^{-7}$</td>
<td>2.7</td>
</tr>
</tbody>
</table>

$^a$The lowest response detected with a signal to noise ratio of 3.
$^*n = 5; 1 \times 10^{-6}$ M

Table 20. Analytical figures of merit obtained with stabilised ruthenium chemiluminescence detection.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Calibration function (peak area, arbitrary units)</th>
<th>$R^2$</th>
<th>LOD (M)$^a$</th>
<th>R.S.D. %$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>$y = 4.97 \times 10^5 x - 57.596$</td>
<td>0.9989</td>
<td>$1.0 \times 10^{-6}$</td>
<td>1.3</td>
</tr>
<tr>
<td>Thebaine</td>
<td>$y = 5.16 \times 10^5 x - 4.078$</td>
<td>0.9996</td>
<td>$1.0 \times 10^{-6}$</td>
<td>1.1</td>
</tr>
<tr>
<td>Heroin</td>
<td>$y = 9.49 \times 10^7 x + 79.766$</td>
<td>0.9882</td>
<td>$5.0 \times 10^{-6}$</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^a$The lowest response detected with a signal to noise ratio of 3.
$^*n = 5; 1 \times 10^{-6}$ M
Table 21. Analytical figures of merit obtained with enhanced permanganate chemiluminescence detection.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Calibration function (peak area, arbitrary units)</th>
<th>( R^2 )</th>
<th>LOD (M)( ^a )</th>
<th>R.S.D. %( ^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>( y = 8.54 \times 10^8 x + 15.827 )</td>
<td>0.9993</td>
<td>( 1.0 \times 10^{-9} )</td>
<td>1.6</td>
</tr>
<tr>
<td>Oripavine</td>
<td>( y = 1.13 \times 10^9 x + 2.657 )</td>
<td>0.9999</td>
<td>( 1.0 \times 10^{-9} )</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\( ^a \)The lowest signal detected with a signal to noise ratio of 3.
\( ^* n = 5, 1 \times 10^{-6} \) M

3.5.1 Application to real samples

The six drug seizure samples were then screened using the multiplexed approach. The chromatograms from the three simultaneous modes of detection were almost identical to those obtained using conventional post-column detection, with only minor differences in sensitivity observed (Figures 102, 103, and 104).

As can be seen in Table 22, application of multiplexed UV-absorbance and chemiluminescence detection generated results that compared favourably. The difference between the two modes of detection ranged between 1.42% and 8.0%. These results are in good agreement with those obtained using conventional post-column detection and ranges previously reported by other researchers [248-254].

Table 22. Concentration of heroin in drug seizure samples obtained with multiplexed UV-absorbance and stabilised tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heroin (percent mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UV-absorbance</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>87</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
</tr>
</tbody>
</table>

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Figure 102. Chromatograms obtained for the analysis of six drug seizure samples with multiplexed UV-absorbance detection. Pink and black traces show sample preparation with and without hydrolysis. Peaks: morphine (MOR), 6-monoacetylmorphine (6-MAM) and heroin (HER).
Figure 103. Chromatograms obtained for the analysis of six drug seizure samples with multiplexed stabilised tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection. Pink and black traces show sample preparation with and without hydrolysis. Peaks: heroin (HER).
Figure 104. Chromatograms obtained for the analysis of six drug seizure samples with enhanced permanganate chemiluminescence detection. Pink and black traces show sample preparation with and without hydrolysis. Peaks: morphine (MOR) and 6-monoacetylmorphine (6-MAM).
4. Conclusions

The rapid separation of *P. somniferum* opiate alkaloids (morphine, oripavine, codeine, thebaine, heroin and papaverine) was achieved in six minutes using conventional HPLC with post-column UV-absorbance or chemiluminescence detection. All of the alkaloids were detectable with UV-absorbance, whilst greater selectivity was imparted via the use of the enhanced permanganate reagent (morphine and oripavine) and the stabilised tris(2,2′-bipyridine)ruthenium(III) reagent (codeine, thebaine and heroin).

The combination of these two stabilised chemiluminescence reagents not only provides the basis of a rapid screening test for heroin, but proved highly compatible with the extended analysis times inherent to HPLC procedures and generated limits of detection comparable to or superior than those previously reported for the *P. somniferum* opiate alkaloids.

The addition of an annular frit to the end of the chromatographic column which enabled segmentation of the column eluate between three unique modes of detection (UV-absorbance and two chemiluminescence reagents) resulted in minor losses of sensitivity (less than an order of magnitude) whilst significantly reducing the sample and solvent consumption, waste generation and total analysis time. Furthermore, application of the multiplexed UV-absorbance and chemiluminescence detectors for the post-column determination of opiate alkaloids in drug seizure samples, resulted in good agreement with values obtained using conventional post-column detection.
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