Extending the Analytical Utility of Acidic Potassium Permanganate Chemiluminescence

by

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ii. List of Publications


iii. Abstract

Chemiluminescence – the production of light as a result of a chemical reaction – is a useful analytical tool because over a wide range the emitted light is proportional to the concentration of the analyte. Adrenergic amines, extracted from *Citrus aurantium* (bitter orange) and commonly used in weight-loss pharmaceuticals, were found to elicit light upon reaction with acidic potassium permanganate. A fast, reproducible method for the determination of these amines in dietary supplements was developed using HPLC coupled with chemiluminescence detection. The amines were quantified in five weight-loss supplements.

During a study on the stability of acidic potassium permanganate, a 6-month old solution of the reagent, which had been exposed to light, was in some cases shown to elicit a greater chemiluminescence signal than a freshly prepared solution, by up to 2 orders of magnitude, prompting further investigation. Manganese(II), formed by the reduction of manganese(VII) over time and in the presence of light, was found to be responsible for the observed enhancement. Addition of manganese(II) to the permanganate reagent was shown to improve sensitivity and compatibility with flow analysis techniques, such as flow injection analysis and liquid chromatography.

The reduction of manganese(VII) by the addition of a manganese(II) salt produced a manganese(III) species. The presence of sodium polyphosphates stabilizes the manganese(III) species and prevents it from forming colloidal
manganese(IV). This reaction requires a minimum of 12 hours to equilibrate, but the use of sodium thiosulphate to reduce potassium permanganate has been shown to be a viable alternative, providing a near-instantaneous method for preparation of the reagent.

Analyte structure was found to influence the observed chemiluminescence intensity, with a preference for ortho- and para- substituted phenols, aminophenols and phenylenediamines over the meta- substituted isomers, which were oxidised at a significantly slower rate by potassium permanganate. A considerable enhancement in the responses for these compounds was achieved, between 50 and 100 fold in some cases, by increasing the concentration of manganese(III) in the potassium permanganate reagent. This was done by reducing the permanganate either with manganese(II) salts or sodium thiosulphate.

The work presented in this thesis has resulted in the development of a new analytical technique for the determination of adrenergic amines in weight-loss supplements. Additional studies into the fundamental chemistry underlying the chemiluminescence reactions with acidic potassium permanganate have led to the ability to fine-tune the selectivity of potassium permanganate reactions toward a certain type of compound, or even a specific positional isomer. Greater understanding of the reaction kinetics have resulted in superior figures of merit for existing procedures, and extended this mode of detection to new analytical applications.
Chapter 1

Introduction

1. Chemiluminescence

2. Acidic potassium permanganate chemiluminescence
   1. Historical considerations
   2. Mechanistic considerations
   3. Analytical applications

3. Instrumentation
   1. Flow injection analysis
   2. Stopped flow analysis

4. Project aims
1. Chemiluminescence

Chemiluminescence is, simply stated, the emission of light from a chemical reaction. Chemical reactions are always accompanied by energy changes; usually excess energy is released as heat, however, in the case of chemiluminescence, it is dissipated as light at wavelengths ranging from near-ultraviolet to near-infrared (200 – 2700 nm). 

In chemiluminescence the reaction between compounds A and B will form a product or intermediate, C, in an electronically excited state, C* (figure 1a). Relaxation through photon emission then returns the excited state species back to its ground state. Alternatively, chemiluminescence can be achieved through an indirect route (figure 1b), where an excited state intermediate transfers its excess energy to a suitable fluorophore, D*, which can then undergo its fluorescence emission.

\[
\begin{align*}
A + B & \rightarrow C^* \\
C^* & \rightarrow C + \text{light} \\
C^* & \rightarrow D^* \\
D^* & \rightarrow D + \text{light}
\end{align*}
\]

(a) (b)

**Figure 1:** With direct chemiluminescence (a), a chemical reaction yields an excited state species, C*, which emits a photon upon relaxation. Indirect chemiluminescence (b), the excited state, C*, transfers its energy to a fluorophore, D*, which then returns to ground state through light emission.
Chemiluminescence reactions can be analytically useful because the light emission is dependent on the concentration of the reactants, the quantum yield of the emitting species and the reaction kinetics\(^1\). This relationship is mathematically described in equation 1. If the physical and environmental factors, to which chemiluminescence reactions are sensitive to (such as temperature, pH, solution mixing) are well controlled, then the emission intensity is directly proportional to the reaction rate, and also to the concentration of the analyte, thereby enabling analytical quantification of unknown concentrations\(^3,4\).

\[
I_{CL} = \phi_{CL} \frac{dP}{dt} = \phi_{EX} \phi_{EM} \frac{dP}{dt}
\]

**Equation 1:** Mathematical relationship between emission intensity \((I_{CL})\), quantum yield \((\phi_{CL})\) and the rate of the chemical reaction \((dP/dt)\), where \(P\) is product and \(t\) is time. The quantum yield \((\phi_{CL})\) is determined by the quantum excitation \((\phi_{EX})\) and quantum emission \((\phi_{EM})\) yields\(^1\).

Chemiluminescence detection offers high sensitivity, selectivity and can be relatively cheap and robust\(^1,5,6\). A further advantage is the facility to generate light without the need for an additional source of electromagnetic radiation, thus reducing the background-to-noise and signal-to-noise ratios, and improving the limit of detection\(^2\). Finally, the compatibility of this mode of detection with other analytical techniques, such as high performance liquid
chromatography (HPLC)$^{7-10}$ and electrophoresis$^{9,11}$ further extends its usefulness. The combination of these techniques may, for example, lead to faster separations, as the inherent selectivity of chemiluminescence detection may produce simplified chromatograms$^{12-15}$. For example, the use of acidic potassium permanganate chemiluminescence with HPLC separation has resulted in chromatograms which had less interference in the region of interest for the determination of Sympetaphine and other adrenergic amines in weight-loss supplements$^{12}$.

The chemiluminescence phenomenon can be observed in the solid-phase, liquid-phase, gas-phase, or it can be electrogenerated$^{4,16}$. The use of solid-phase chemiluminescence is relatively rare and usually involves the formation of excited state aldehydes or ketones$^{17}$. Gas-phase chemiluminescence has been found to be particularly useful in the field of atmospheric analysis, where, for example, the reaction of ozone and nitric oxide forms an excited-state nitrogen dioxide, which returns to ground state through emission of radiation$^{18}$. Gas-phase chemiluminescence has also been applied for the detection of nitrogen compounds, sulphur compounds and hydrocarbons$^{18}$. A number of reagents have been identified as being capable of inducing liquid-phase chemiluminescence, including acidic potassium permanganate, tris(2,2'-bipyridyl)ruthenium(II), peroxoalates and luminol$^{19}$. The above mentioned reagents have been utilised in the analysis of both organic and inorganic species.
Chemiluminescence can be observed in the biological world where it is referred to as bioluminescence. Perhaps the most obvious example is the firefly, but it is also seen with some species of bacteria, protozoa and crustacea\textsuperscript{20}.

2. Acidic potassium permanganate chemiluminescence

2.1 Historical background

The use of potassium permanganate as a chemiluminescence reagent can be traced back to the early 20\textsuperscript{th} century. Harvey\textsuperscript{21} first reported the oxidation of pyrogallol with acidic potassium permanganate in 1917. Three years later Grinberg\textsuperscript{22} published his findings on the oxidation of pyrogallol with acidic permanganate. In 1925 Kautsky and Neitzke\textsuperscript{23} investigated the oxidation of siloxane by both potassium permanganate and ozone. During the 1930’s the chemiluminescence of glucose with potassium permanganate was reported by Audubert\textsuperscript{24-26}.

Mizuno\textsuperscript{27} and colleagues described “extra-weak” chemiluminescence, observed using photon counting, for the reaction between oxalic acid and potassium permanganate. In 1972 the reagent was used for the analytical determination of gas resistance in plants\textsuperscript{28}. Three years later this reagent was applied in the quantification of trace concentrations of atmospheric sulphur dioxide\textsuperscript{29}. In 1977 and 1978 Lebedev\textsuperscript{30-34} et al. reported the
chemiluminescence accompanying the oxidation of various amino acids by potassium permanganate.

Analytical applications using acidic potassium permanganate specifically for the detection of organic species first emerged in the mid-1980's\(^{35,36}\). Since that time uses of acidic potassium permanganate chemiluminescence have significantly increased, particularly with regard to the determination of organic analytes. A critical and comprehensive review of the development of this reagent was published in 2001 by Hindson\(^{37}\) and colleagues. A more recent review of the reagent was published by Adcock\(^{38}\) and co-workers.

### 2.2 Mechanistic considerations

The function of acidic potassium permanganate in analytical chemistry has grown throughout the last few decades, from only one application published in 1975, to 35 publications in 2005 describing analytical applications with this reagent. Throughout that time there has been much debate on the mechanism of these types of reactions, with numerous hypotheses on the nature of the emitting species. The proposals have included the following excited state intermediates; singlet oxygen, nitrogen, nitric oxide, sulphur dioxide and fluorescent intermediates from the analyte\(^{14,39-49}\). Additionally, numerous researchers have suggested an excited state manganese(II) or a complex thereof as the emitter\(^{31,32,34,50}\). In 2002 the first chemical and spectroscopic evidence showing light emission did indeed arise from an excited-state manganese(II) species was reported\(^{50}\). This was later confirmed
in the work of Adcock$^{51}$ et al. Recently, Hindson$^{52}$ and co-workers revealed a mechanism for the formation of the excited-state manganese(II)$^*$. Additional discussion on the mechanism of the reaction can be found in chapter 4 of this thesis.

2.3 Analytical Applications

Acidic potassium permanganate chemiluminescence has been applied as an analytical tool in various fields, including the agricultural, pharmaceutical and food and wine industries. In the pharmaceuticals, for example, this method of detection has been utilised in the detection of opiate alkaloids in process streams, with and without prior sample separation$^{53,54}$. The detection of morphine in urine$^{55}$ and maggots$^{56}$ has also been reported. Penicillin-type antibiotics, including penicillin G, phenoxyethylpenicillin, amoxicillin and ampicillin, have been quantified in tablets and pharmaceutical formulations$^{42,57-61}$. The numerous analytical applications of this reagent are extensively discussed in a comprehensive review by Adcock$^{38}$ et al.

3. Instrumentation

3.1 Flow injection analysis

Flow injection analysis, incorporating a continuous-flow methodology, was first described by Ruzicka and Hansen$^{62}$. Shortly after, Stewart$^{63}$ and co-
workers utilised FIA for the rapid analysis of trypsin, with sample rates as high as 120 samples per hour.

A very simple instrument setup (figure 2) requires continuous propulsion of the reagent, which can usually be achieved by the use of a syringe or a peristaltic pump. The use of a syringe provides a smoother transport of the reagent through the tubing; however the sampling rate is compromised due to the need to periodically refill the syringe. A peristaltic pump, on the other hand, uses a series of rollers to push a fluid through the tubing resulting in a near pulse-free, continuous flow of a solution through the tubing. Both methods of propulsion allow for reproducible timing, one of the key foundations in the operation of flow injection systems.

![Diagram of FIA setup](image)

**Figure 2:** A schematic of a simple FIA setup. The sample is injected into the flowing stream and the mixture passes through a reactor before reaching the detector (D) and finishing in the waste (W).
A sample can be introduced to the constantly flowing carrier stream directly, as a pulse or plug of liquid. Alternatively, the use of multi-port valves can divert the flow of the carrier stream through the sample loop, picking up an accurate volume of sample without disturbing the flow. In this instance the sample volume is determined by the size of the sample loop on the multi-port valve\textsuperscript{66}. The reagent and sample zones are then propelled through the mixing coil before reaching the detector, then finally the solution is discarded into waste, and a new sample can be introduced for analysis\textsuperscript{57}.

The inherent simplicity of this methodology offers several advantages, including an instrument system which can be straightforward, low-cost and portable, whilst affording rapid and reproducible results\textsuperscript{66,67}. The technique does however rely on consistent and reliable solution propulsion, which results in dispersion\textsuperscript{65}. Sample dispersion, or band broadening, is caused by two factors; (i) convection, whereby the centre of the sample moves more quickly than the liquid adjacent to the walls of the tubing, and (ii) diffusion, which occurs either perpendicular or parallel to the flow direction. Dispersion, which is mathematically defined as the ratio of the sample concentration injected into the carrier stream and the peak concentration at the detector, is influenced by four controllable variables; flow rate, tube length, tube diameter and sample volume\textsuperscript{65}.

Numerous detectors can be coupled with FIA methodologies, including atomic absorption and emission spectrometers, fluorometers, spectrophotometers and photometers\textsuperscript{68-70}. For example, in 2004, Ruedas Rama\textsuperscript{71} and colleagues
coupled flow injection analysis with bead-injection spectroscopy for the
determination of promethazine and trifluoperazine. Flow injection analysis
with visible spectroscopy was applied in the determination of insolubles in
diesel lubricating oil\textsuperscript{72}. Determination of platinum, palladium and rhodium
was achieved by utilising FIA with on-line solid phase extraction and flame
atomic absorption spectroscopy\textsuperscript{73}. Recently a simple method for the
determination of pyrite oxidation rates was published by Osborne and co-
workers\textsuperscript{74}. The method utilised spectroscopy with flow injection analysis to
achieve its goal.

Flow injection analysis is a well established methodology, with numerous
reviews published on the analytical technique. In 2008 Ruzicka and Hansen\textsuperscript{75}
reviewed the development of flow injection analysis in the time since the
publication of their book on the topic\textsuperscript{67}, twenty years earlier. Spectrometric
determination of pharmaceuticals utilising flow injection analysis is described
in the work of Tzanavaras and Themelis\textsuperscript{76}. An in-depth review of flow injection
analysis and its analogue, sequential injection analysis, with respect to
chemiluminescence determination of pharmaceuticals was prepared by
Mervartova\textsuperscript{68} and colleagues.

3.2 Stopped flow analysis and kinetics

In flow analysis\textsuperscript{77,78} a sample and reagent are simultaneously propelled into
the reaction chamber, where they are held, and the subsequent reaction is
detected. This method of analysis results in the acquisition of a signal as a
function of time, thereby obtaining information on the kinetic profile of the reaction, as the physical process of dispersion is limited to molecular diffusion only. As a continuous flow is not required with this method, a syringe pump is usually used to propel the sample and reagents. With proper setup, this ensures that the two solutions are stopped inside the reaction chamber, and rapid mixing is achieved as a result of the high rate and pressure produced by the pump. A typical chemiluminescence signal as a function of time rises rapidly as the reactants are combined. Once the mixing is complete the emission reaches a maximum and then gradually decays (figure 3). Quantitation from this signal is achieved either by measuring peak height at maximum emission, or peak area of the signal over a fixed period of time. The reaction profile is dependent on the rate of formation of the excited state intermediate and the rate of decay for said intermediate.

**Figure 3:** Typical signal response as a function of time profile observed with flow analysis techniques.
The kinetic profile of a reaction is indicative of the chemistry involved, thus it can be used not just to optimise the chemiluminescence response\(^\text{79}\), but also as an analytical technique\(^\text{81}\). For example, by obtaining the complete reaction profile of each analyte with a specific reagent, and assuming the reaction between the reagent and each analyte is independent; the kinetic profile of the mixture of analytes is the sum of each individual profile. Therefore analytical determination of individual analytes may be possible through appropriate mathematical modelling, allowing for simultaneous determination without the need for sample separation.

4. Project aims

New analytical applications utilising potassium permanganate were investigated. In addition to this, further studies into the fundamental chemistry of potassium permanganate chemiluminescence were undertaken. An understanding of the structural relationship between an analyte and the chemiluminescence intensity with acid potassium permanganate was sought. The role of manganese(II) in this reaction, and its potential to selectively tune the reaction towards certain types of analytes, or structural isomers of the same compound, which can be exploited for future applications of acidic potassium permanganate chemiluminescence, was also studied.
Chapter 2

Determination of adrenergic amines in weight-loss supplements using HPLC coupled with acidic potassium permanganate chemiluminescence

1. Introduction

2. Experimental
   1. Reagents, standards and samples
   2. Instrumentation
   3. FIA manifolds

3. Results and discussion
   1. Preliminary Investigations
   2. Potential interference study
   3. Development of the separation methodology
   4. Quantification of adrenergic amines in weight-loss products

4. Conclusions
1. Introduction

In 2004, the US Food and Drug Administration (FDA) banned the sale of all dietary supplements containing ephedrine alkaloids due to their potential to cause serious, adverse health effects\(^4\). As a result of the FDA prohibition, there has been a sharp increase in the use of ephedrine-free weight-loss nutraceuticals\(^82,83\). A common ingredient of many supplements is the extract of *Citrus aurantium* (bitter orange, Seville orange, sour orange), which contains synephrine and several other phenolic amines\(^83,84\).

Synephrine and ephedrine have a similar chemical structure (figure 4). Both compounds are secondary amines, however ephedrine does not contain a phenolic moiety. Synephrine has been promoted by some supplement manufacturers as a safe alternative to ephedrine, but there are concerns that it may also cause unwanted side effects\(^85,86\).

![Chemical structures of ephedrine and synephrine.](image)

**Figure 4:** Chemical structures of ephedrine and synephrine.
The most active components of the dried fruit of *Citrus aurantium* are synephrine and octapamine, however, other structurally related α-adrenergic agonists – tyramine, *N*-methyltyramine and hordenine – (figure 5) may also be present at lower concentrations\(^{82-84}\). *Citrus aurantium*, a small citrus tree, is primarily used for medicinal purposes, as it is mostly considered too bitter to eat, but in the West the fruit or peel has been used in marmalade and bouquet garni, and as an appetiser\(^ {82}\). The dried fruit has been used for millennia in Asian herbal medicines to increase energy, improve circulation and treat digestive problems\(^ {82,87}\). Essential oils derived from the fruit have also been used in perfumes, liqueurs and sweets\(^ {82}\).

**Figure 5:** Chemical structures of octopamine (O), synephrine (S), tyramine (T) and hordenine (H), α-adrenergic antagonists commonly found in *C. aurantium*.
Due to the sharp increase in the number of weight-loss products containing *Citrus aurantium* extracts and the associated concerns from health professionals, numerous new analytical methods for quantifying synephrine and related adrenergic amines in citrus plant extracts and commercially available supplements have emerged in the scientific literature, including electrophoresis with UV-absorbance and HPLC with UV-absorbance, fluorescence, electrochemical, electrospray ionization mass spectrometry (ESI-MS), tandem mass spectrometry (MS-MS). The wide variety of conditions, sample treatments and detection modes is, in part, due to the target applications, which include a simple rapid screening for process analysis, identification to ascertain legal compliance and quantification of analyte isomers or enantiomers.

Kusu and coworkers determined synephrine and *N*-methylyramine in immature citrus fruits using high performance liquid chromatography (HPLC) coupled with electrochemical detection. Li et al. used UV-absorbance to quantify synephrine and *N*-methylyramine in *Pericarpium citri reticulatae*, post HPLC separation.

Pellati and colleagues resolved synephrine, octopamine and tyramine by reverse phase chromatography, using UV-absorbance detection at 220 nm. Avula and Khan proposed a method for the determination of ephedrine enantiomers and synephrine by high performance capillary electrophoresis. A flow injection method for the sensitive determination of synephrine was
published by Li\textsuperscript{99} \textit{et al.}, based on the enhancement of the chemiluminescence reaction between luminol and hexacyanoferrate(III) under alkaline conditions. This method was applied to the determination of synephrine in urine, plasma and herbal products, however many of these matrices contain interfering species, thus requiring separation prior to detection\textsuperscript{100}.

In this chapter a new analytical method is proposed for the determination of adrenergic amines in weight-loss nutraceuticals containing \textit{C. aurantium} extract. A silica based monolithic column will be utilised to separate the target compounds from interferents. These columns are constructed from a single, porous silica rod. This construction allows greater flow rates of the mobile phase and a reduced backpressure, resulting in a faster separation than previously reported. The proposed method will be coupled with acidic potassium permanganate chemiluminescence, which will provide greater selectivity than UV-Visible absorbance, and a simpler setup than luminol chemiluminescence proposed by Li\textsuperscript{99} \textit{et al.}. 
2. Experimental

2.1 Reagents, standards and samples

The chemiluminescence reagent was prepared by dissolving 1 mM potassium permanganate, purchased from Ajax (Auburn, NSW, Australia), and 1% (w/v) sodium polyphosphate crystal mesh (Sigma-Aldrich; Castle Hill, NSW, Australia) in deionised water. The pH of the reagent was adjusted by the addition of concentrated sulphuric acid (Ajax).

Stock solutions (1 mM) of synephrine, tyramine, and octopamine (Sigma–Aldrich) were prepared in deionised water. Hordenine was synthesised from tyramine using a modified version of the Eschweiler-Clarke N-methylation reaction\textsuperscript{101}. A stock solution of hordenine (1 mM) was prepared by dissolving the solid in deionised water with sonication (10 min).

Herbal extracts and dietary supplements were obtained in Australia and the U.S. Most of the products indicated the amount of *C. aurantium* in the product in terms of dry fruit equivalent. One weight-loss product that listed a *C. aurantium* extract and specified quantities of octopamine, tyramine and hordenine was purchased in the USA. Samples were prepared by dissolving the tablet, capsule or extract in 50 or 250 mL of deionised water, with stirring (and in some cases sonication) for 30 min. Concentrations of the analytes were reduced to within the calibration range by dilution with deionised water. All samples were filtered with 0.45 µm membrane syringe filters.
2.2 Instrumentation

The flow injection analysis manifold consisted of a Gilson Minipuls 3 peristaltic pump from John Morris Scientific (Balwyn, Vic., Australia), bridged PVC tubing and 0.8 mm i.d. PTFE manifold tubing from DKSH (Caboolture, Qld, Australia) and a Valco six-port injection valve from SGE (Ringwood, Vic., Australia) with an 70 µL sample loop (see figure 6, system 3). Samples were injected into a water carrier stream than merged with the acidic potassium permanganate reagent stream at a T-piece, immediately prior to entering a coiled PTFE flow cell positioned against an Electron Tubes Model 9828SB photomultiplier tube (PMT) purchased from ETP (Ermington, NSW, Australia). The PMT was operated at 900 V provided by an Electron Tubes Model PM20D power supply and an Electron Tubes Model C611 voltage divider from ETP. The flow cell, PMT and voltage divider were enclosed in a light-tight housing. The output from the PMT was converted by a Thorn-EMI Model A1 transimpedance amplifier purchased from ETP, and documented with a type 3066 chart recorder from Yokogawa Hokushin Electric (Tokyo, Japan).

The HPLC instrumentation consisted of a Hewlett Packard 1100 series liquid chromatograph equipped with a quaternary pump, solvent degasser system, autosampler and UV–visible absorbance detector from Agilent Technologies (Forest Hill, Vic., Australia). The HPLC was fitted with a Chromolith SpeedROD RP-18 monolithic column and a 5 mm monolithic guard column from Merck (Kilsyth, Vic., Australia) and chemiluminescence detector (as described above)
by replacing the carrier line in the T-piece with the outlet line from the UV-visible absorbance detector. Thus, the column eluent was propelled sequentially through the two detectors. A Hewlett Packard analogue to digital interface box from Agilent Technologies was used to make the signal from chemiluminescence detector compatible with the Hewlett Packard Chemstation software used to control the instrument.

The analytes were separated using an aqueous solution of trifluoroacetic acid (pH 2.5) for 5 min. Remaining sample components were then eluted from the column using 30% methanol and 70% trifluoroacetic acid (pH 2.5) solution for 15 min. Mobile phases were filtered through 0.45 μm membranes. A flow rate of 1 mL min⁻¹ and an injection volume of 10 μL were used for all experiments.

2.3 FIA manifolds

The FIA manifolds for the four chemiluminescence systems consisted of components as described in section 2.2 of this chapter. The specific setup of each system is highlighted in figure 6.

System 1 consisted of 0.08 mM luminol (3-aminophthalhydrazide, Sigma-Aldrich, Castle Hill, NSW, Australia) in 0.5 mM sodium hydroxide (Ajax, Auburn, NSW, Australia); 0.1 mM potassium hexacyanoferrate(III) (potassium ferricyanide, Ajax).
The reagent used in system 2 was prepared as follows; 0.8 mM cerium(IV) sulphate (BDH, Poole, UK) in 1.5 M nitric acid (ChemSupply, Gillman, SA, Australia); 20 μM Rhodamine B (BDH).

System 3 consisted of 1 mM potassium permanganate (Ajax) in 1% (w/v) sodium polyphosphate (Sigma-Aldrich). This solution was adjusted to pH 2.5 with concentrated sulphuric acid (Ajax).

System 4 used a 0.5 mM solution of manganese(IV) solution prepared using potassium permanganate, sodium formate (BDH) and 3.0 M orthophosphoric acid (BDH), as previously described\textsuperscript{102}; 0.5 mol/L formaldehyde (Ajax).
Figure 6: FIA manifolds used to instigate chemiluminescence with alkaline luminol (system 1), Rhodamine B/acidic cerium(IV) (system 2), acidic potassium permanganate (system 3) and manganese(IV) (system 4). The peristaltic pump (p), injector valve (v), detector (d) and waste (w) are also shown.
3. Results and Discussion

3.1 Preliminary investigations

Previous works with potassium permanganate chemiluminescence, reviewed by Adcock\textsuperscript{38} \textit{et al.} have shown that many phenolic species with amine moieties elicit light with this reagent. Adrenergic amines – synephrine, octopamine, tyramine, hordenine – (figure 5) contain both phenol and amine groups, and thus it was anticipated that these compounds would also react with potassium permanganate and liberate light. A simple two-line flow injection manifold, (figure 6 - system 3) was used to investigate the chemiluminescence of the adrenergic amines.

The analytes were injected into the water carrier stream prior to merging with acidic potassium permanganate. A chemiluminescence signal was observed with all four analytes of interest. During optimization it was observed that the maximum signal for tyramine with acidic permanganate occurred at the highest pH tested (pH 3), while synephrine, octopamine and hordenine elicited maximum response at pH 2.5 (figure 7). The chemiluminescent response of all four amines was reduced by approximately 50% at pH 2.0, compared to the higher pH’s. The difference in the chemiluminescence response of tyramine at pH 2.5 and 3.0 was insignificant, thus it was decided that the permanganate reagent would be adjusted to pH 2.5 for the determination of all four amines.
**Figure 7**: Effects of pH on the chemiluminescent signal of adrenergic amines.

The consequence of flow rate on the chemiluminescence was investigated. A series of flow rates between 3.0 mL min\(^{-1}\) and 9.0 mL min\(^{-1}\) were applied and the peak height for each adrenergic amine was observed. Flow rate was found to have a minimal effect on the signal intensity for these amines, suggesting a similar, slow rate of oxidation of the four analytes by permanganate. The conditions used to induce chemiluminescence are summarized in table 1, and
the analytical figures of merit for these adrenergic amines are shown in table 2.

<table>
<thead>
<tr>
<th>Oxidant concentration</th>
<th>$1 \times 10^{-3}$ M Potassium permanganate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enhancer concentration</td>
<td>$1%$ (w/v) sodium polyphosphate</td>
</tr>
<tr>
<td>pH</td>
<td>2.5 adjusted by addition of concentrated sulphuric acid</td>
</tr>
<tr>
<td>Flow rate</td>
<td>3.0 mL/min total flow rate</td>
</tr>
</tbody>
</table>

Table 1: Reagent conditions developed for acidic potassium permanganate chemiluminescence with adrenergic amines.

<table>
<thead>
<tr>
<th>Adrenergic amine</th>
<th>Detection limit (3$\sigma$)</th>
<th>Linear range</th>
<th>Correlation coefficient ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synephrine</td>
<td>$1 \times 10^{-8}$ M</td>
<td>$1 \times 10^{-7}$ M – $1 \times 10^{-4}$ M</td>
<td>0.9972</td>
</tr>
<tr>
<td>Octopamine</td>
<td>$5 \times 10^{-7}$ M</td>
<td>$1 \times 10^{-7}$ M – $1 \times 10^{-4}$ M</td>
<td>0.9985</td>
</tr>
<tr>
<td>Tyramine</td>
<td>$1 \times 10^{-8}$ M</td>
<td>$1 \times 10^{-7}$ M – $1 \times 10^{-4}$ M</td>
<td>0.9983</td>
</tr>
<tr>
<td>Hordenine</td>
<td>$5 \times 10^{-8}$ M</td>
<td>$1 \times 10^{-7}$ M – $1 \times 10^{-4}$ M</td>
<td>0.9915</td>
</tr>
</tbody>
</table>

Table 2: Chemiluminescence response of adrenergic amines with acidic potassium permanganate, using flow injection analysis.
3.2 Potential interference study

As mentioned earlier in this chapter, a flow injection method for the sensitive determination of synephrine has been reported by Li\textsuperscript{99} \textit{et al.}. The method was based on the enhancement of the alkaline luminol and hexacyanoferrate(III) reaction. The same research group also quantified synephrine in citrus fruit extracts, blood and urine using flow injection analysis based on a reaction with cerium(IV) and Rhodamine B in acidic solution\textsuperscript{103}. In both studies the potential interference from uric acid, inorganic salts and sugars was investigated, but the numerous phenolic compounds present in \textit{C. aurantium} extracts and biological fluids were not examined.

In this chapter, the initial investigation into the chemiluminescence of adrenergic amines (synephrine, octopamine, tyramine and hordenine) has shown all four amines elicit light upon reacting with acidic potassium permanganate. Consequently, quantification of synephrine in citrus fruit extracts using flow injection analysis with acidic potassium permanganate chemiluminescence detection would likely cause significant error in some cases, as the observed chemiluminescence signal could be due to any one of the four adrenergic amines.

The adrenergic amines were examined with the two techniques proposed by Li\textsuperscript{99,103} and coworkers – flow injection analysis using (i) alkaline luminol / hexacyanoferrate(III) (FIA system 1) and (ii) acidic cerium(IV) / Rhodamine B
reagents (FIA system 2), as well as with Mn(IV) / formaldehyde (FIA system 4) and acidic potassium permanganate (FIA system 3). Preliminary results, reported in section 3.2 of this chapter, have shown potassium permanganate chemiluminescence not to be sufficiently selective to quantify synephrine, without a sample separation step, in a matrix where other potential interferants may be present. Selective determination of synephrine in complex samples, such as blood and urine, would require a chemiluminescence reagent that is very selective toward synephrine and has a minimal response with other potential interferants, such as urinary metabolites of epinephrine or dopamine\textsuperscript{100}. In addition to potassium permanganate, manganese(IV) and the reagents suggested by Li\textsuperscript{99} et al and Wang\textsuperscript{103} et al were investigated to establish if any of the reagents had a high selectivity toward synephrine over potential interferants.

As illustrated by the data in table 3, synephrine, octopamine and tyramine produce chemiluminescence signals with all four reagents mentioned above. Phenolic amines (such as octopamine and tyramine) are present in \textit{citrus aurantium} extracts and could interfere in the analytical determination of synephrine using simple flow injection methodology, particularly with some herbal products which contain these anilines added as separate ingredients.

Additional phenolic compounds selected for comparison (table 3) were either commonly found in the types of samples analysed in the proposed
methods for the determination of synephrine\textsuperscript{12,99,103}, or previously shown to give a significant response with one or more of the four chemiluminescence reagent systems\textsuperscript{47,102,104-109}. The neurotransmitters dopamine and serotonin have been studied with permanganate\textsuperscript{47,106,107} and luminol\textsuperscript{108-110} systems and also with soluble manganese(IV)\textsuperscript{102,111}. Previous studies have identified protocatechuic acid and caffeic acid as strong enhancers of the luminol / hexacyanoferrate(III) reaction.

Homovanillic acid, vanilmandelic acid and 5-hydroxyindoleacetic acid are urinary metabolites of epinephrine, dopamine and serotonin. These compounds have been reported to respond to acidic potassium permanganate and luminol / hexacyanoferrate(III) reagents\textsuperscript{112-114}.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Luminol ( K_3Fe(CN)_6 )</th>
<th>Ce(IV)Rhodamine ( B )</th>
<th>( KMnO_4 ) polyphosphates</th>
<th>Mn(IV) formaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synephrine</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Tyramine</td>
<td>27</td>
<td>116</td>
<td>133</td>
<td>93</td>
</tr>
<tr>
<td>Octopamine</td>
<td>154</td>
<td>99</td>
<td>109</td>
<td>99</td>
</tr>
<tr>
<td>Dopamine</td>
<td>79</td>
<td>14</td>
<td>302</td>
<td>47</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>*</td>
<td>1</td>
<td>88</td>
<td>78</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>*</td>
<td>20</td>
<td>146</td>
<td>77</td>
</tr>
<tr>
<td>Vanilmandelic acid</td>
<td>35</td>
<td>43</td>
<td>73</td>
<td>102</td>
</tr>
<tr>
<td>Serotonin</td>
<td>*</td>
<td>4</td>
<td>2375</td>
<td>102</td>
</tr>
<tr>
<td>5-hydroxyindoleacetic acid</td>
<td>*</td>
<td>5</td>
<td>708</td>
<td>125</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>*</td>
<td>5</td>
<td>135</td>
<td>85</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>*</td>
<td>13</td>
<td>94</td>
<td>74</td>
</tr>
</tbody>
</table>

*Inhibition of background signal

**Table 3**: Chemiluminescence signal for certain phenolic compounds \( (5 \times 10^{-5} \text{ M}) \) with four different reagent systems, relative to synephrine \( (100\% \text{ signal}) \) for each reagent system.
The data presented in table 3 shows that the four reagent systems investigated offer a sensitive means to detect synephrine, and other compounds of interest, however none of the four reagent systems studied show sufficient preference to selectively determine synephrine in herbal weight-loss products or biological fluids using FIA methodology alone. To selectively quantify adrenergic amines in complex samples, such as weight-loss supplements, it is necessary to physically separate the sample components prior to detection.

3.3 Development of HPLC separation methodology

To achieve the necessary sample separation high performance liquid chromatography was coupled with chemiluminescence detection, as shown by the simple schematic in figure 8. Initial separation method of the adrenergic amine standards involved an isocratic separation with deionised water adjusted to pH 2.0 with trifluoroacetic acid (TFA), and methanol, as the eluents (97% H₂O-TFA (v/v) / 3% methanol (v/v)). Trifluoroacetic acid was added to water to act as a buffer, preventing the build-up of salt residues on the column.

A monolithic column was chosen due to the characteristics of this type of column. Based on the design, consisting of a single, highly porous silica rod, a monolithic column produces less back pressure, and is therefore capable of running at higher flow rates, allowing faster separations. The initial
separation method was loosely based on the work by Costin\textsuperscript{15} and coworkers on opium alkaloid separation using a monolithic column, as this group used a monolithic column of similar dimensions to separate compounds which are comparable in size and structure to adrenergic amines. Based on this method the mobile phase was eluted at 3 mL/min and a 5 minute run time was used.

![Diagram](image_url)

**Figure 8:** Simple schematic of a setup used for HPLC separation coupled with CL detection.

This initial separation (see figure 9) using the above methodology was complete within 1 minute. Tyramine and hordenine were well resolved, eluting at 0.7 min and 0.95 min, respectively, but octopamine and synephrine were not completely separated. Synephrine is claimed to be the main ingredient responsible for weight loss by many of the supplement
manufacturers, it is therefore expected that synephrine would be present in the nutraceuticals at higher concentration than the other adrenergic amines. Consequently it is pertinent to achieve a complete separation of synephrine from the other α-antagonists commonly found in *Citrus aurantium* extracts.

![Graph showing separation of adrenergic amines](image)

**Figure 9:** Separation of adrenergic amines octopamine (1), synephrine (2), tyramine (3) and hordenine (4). Column used was Chromolith SpeedROD RP-18.

The methodology was modified to completely resolve the synephrine and octopamine peaks (peaks 1 and 2 in figure 9). The amount of methanol used in the mobile phase was reduced to 2% (v/v) to increase the retention of
the amines on the column. This did not have a significant impact on the resolution. Methanol was then removed from the mobile phase and the flow rate decreased to 1 mL/min, which resulted in sufficient resolution of all four peaks (figure 10). The method, including the post-sample column wash and re-equilibration is reviewed in table 4.

<table>
<thead>
<tr>
<th>Column</th>
<th>Chromolith™ SpeedROC RP-18, 50 x 4.6 mm id with 5 mm guard column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluent A</td>
<td>Deionised water, adjusted to pH 2 with trifluoroacetic acid (TFA)</td>
</tr>
<tr>
<td>Eluent B</td>
<td>Methanol</td>
</tr>
<tr>
<td>Injection</td>
<td>10 µL</td>
</tr>
<tr>
<td>Sample run conditions</td>
<td>100% eluent A, isocratic 5 minute run</td>
</tr>
<tr>
<td>Post run conditions</td>
<td>Total post run time is 20 minutes, consisting of a column flush (70% A / 30% B, 15 minutes, isocratic) and a re-equilibration time (100% A, 5 minutes)</td>
</tr>
<tr>
<td>Analyte</td>
<td>Octopamine, Syneprine, Tyramine, Hordenine</td>
</tr>
<tr>
<td>Elution time</td>
<td>1.1 min, 1.6 min, 2.4 min, 4 min</td>
</tr>
</tbody>
</table>

Table 4: HPLC conditions used to achieve separation of four adrenergic amines.
**Figure 10:** HPLC separation of adrenergic amines on the Chromolith SpeedROD RP-18 column with a 5 mm guard column. The separation of octopamine (O), synephrine (S), tyramine (T) and hordenine (H) is achieved in 4.5 min.

Baseline resolution was achieved within a 5 minute run time, which at the time, to the best of my knowledge, was the fastest elution time reported for these amines (see table 5). The elution order of the adrenergic amines is influenced by their polarity. The most polar (octopamine) elutes first, followed by synephrine, tyramine and finally hordenine.
<table>
<thead>
<tr>
<th>Separation conditions</th>
<th>Analytes</th>
<th>Separation time</th>
<th>Method of detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18 column. Isocratic conditions (aqueous solution of citric acid and sodium dihydrogen phosphate)</td>
<td>Synephrine, N-methylyramine, (norepinephrine)</td>
<td>16 min</td>
<td>Electrochemical</td>
<td>94</td>
</tr>
<tr>
<td>C18 column. Isocratic conditions (aqueous solution of citric acid and sodium dihydrogen phosphate; pH 3)</td>
<td>Octopamine, synephrine, tyramine</td>
<td>8 min</td>
<td>UV-absorbance (220 nm)</td>
<td>98, 98</td>
</tr>
<tr>
<td>Ion-pair chromatography on a C18 column. Gradient elution with (i) aqueous solution of SDS; (ii) aqueous phosphoric acid with SDS; (iii) mixture of acetonitrile and methanol</td>
<td>Octopamine, synephrine, tyramine, (+3 ephedrine alkaloids)</td>
<td>18 min (+8 min)</td>
<td>UV-absorbance (210 nm)</td>
<td>92</td>
</tr>
<tr>
<td>C18 column. Gradient elution with sodium acetate buffer (pH 5.5) and acetonitrile</td>
<td>Octopamine, synephrine, N-methylyramine, hordenine, methoxysynephrine, (+20 flavonoids)</td>
<td>17 min (+33 min)</td>
<td>UV-absorbance (254, 280 and 330 nm)</td>
<td>84</td>
</tr>
<tr>
<td>C18 column. Isocratic conditions using room-temperature ionic liquids</td>
<td>Octopamine, synephrine, tyramine</td>
<td>8 min</td>
<td>UV-absorbance (273 nm)</td>
<td>93</td>
</tr>
<tr>
<td>Monolithic C18 column. Isocratic conditions (aqueous solution of trifluoroacetic acid; pH 2.0)</td>
<td>Octopamine, synephrine, tyramine, hordenine</td>
<td>5 min</td>
<td>UV-absorbance (280 nm) and chemiluminescence</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a* Not including post analysis and re-equilibration time  
*b* Internal standard  
*c* Time required to elute additional analytes  

**Table 5**: HPLC conditions for the separation of adrenergic amines in *C. aurantium*.  

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3.4 Quantification of adrenergic amines in weight-loss samples

Weight-loss supplements often have a complex sample matrix. For example, five weight-loss products were purchased and noted to also contain dry extracts of *Paullinia cupana* seed (Guarana), *Salix alba* stem bark, *Camillia sinensis* (Green tea) in addition to *citrus aurantium* (Bitter orange) dry fruit extract. The extensive list of additives in any single weight-loss supplement can introduce numerous interferences, thus making the separation and quantification of any individual compound a challenging assignment.

By utilizing the selective nature of high performance liquid chromatography with chemiluminescence detection, simpler chromatograms can be produced, enabling accurate quantification of compounds of interest in complex matrixes, as shown in figure 11.
Figure 11: An example of (a) UV-absorbance (a.u) at 280 nm and (b) chemiluminescence (mV) detection for the HPLC determination of synephrine (S) and hordenine (H) in dietary supplements.

Synephrine is observed in both the UV and CL chromatogram (largest peak shown in figure 11). The synephrine peak observed with UV detection is not in perfect alignment with the same peak detected by chemiluminescence due to the distance between the column end and the two detectors. The separated solution first enters the UV detector before being propelled into the chemiluminescence detector; therefore the UV peaks are always detected slightly ahead of the chemiluminescence peaks. The UV chromatogram also
shows trace amounts of several other compounds commonly found in these nutraceuticals, seen in figure 11 as minor peaks in the chromatogram. Furthermore, the elution of hordenine (at 4 minutes) and an interferant is noted in the UV chromatogram. The interferant does not elicit light upon reacting with acidic potassium permanganate, thus it is not observed in the chemiluminescence chromatogram, and only the peak for hordenine is observed.

The chemiluminescence chromatogram also shows a clean peak for synephrine. The minor peaks, visible in the UV chromatogram, are not observed with chemiluminescence detection. The intrinsic selectivity of chemiluminescence detection results in the generation of simpler chromatograms from complex samples, as illustrated by figure 11, and in so doing improving the selectivity of this mode of detection.

Two calibration functions for each amine were obtained, where one function covered higher concentrations and the other covered lower concentrations (shown in figure 12). This was performed with both chemiluminescence and ultraviolet absorbance detection. The analytical figures are summarised in table 6.
Figure 12: Log-Log calibration functions for synephrine and octopamine at higher concentrations (between $1 \times 10^{-3}$M and $1 \times 10^{-6}$M) and lower concentrations (between $1 \times 10^{-8}$ M and $1 \times 10^{-9}$ M).
<table>
<thead>
<tr>
<th>Chemiluminescence</th>
<th>Synephrine</th>
<th>Octopamine</th>
<th>Tyramine</th>
<th>Hardenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>$Y = 2.55x - 1.33$  \ ($R^2 = 0.9983$)</td>
<td>$Y = 2.95x - 1.21$  \ ($R^2 = 0.9985$)</td>
<td>$Y = 6.77x - 2.29$  \ ($R^2 = 0.9990$)</td>
<td>$Y = 3.12x - 2.34$  \ ($R^2 = 0.9937$)</td>
</tr>
<tr>
<td>High</td>
<td>$Y = 3.23x - 17.89$  \ ($R^2 = 0.9981$)</td>
<td>$Y = 3.49x - 11.65$  \ ($R^2 = 0.9991$)</td>
<td>$Y = 5.73x + 19.4$  \ ($R^2 = 0.999$)</td>
<td>$Y = 3.49x - 6.69$  \ ($R^2 = 0.9992$)</td>
</tr>
<tr>
<td>UV absorbance</td>
<td>$Y = 3.41x - 0.49$  \ ($R^2 = 0.9991$)</td>
<td>$Y = 3.14x - 0.07$  \ ($R^2 = 0.9989$)</td>
<td>$Y = 5.34x - 2.38$  \ ($R^2 = 0.997$)</td>
<td>$Y = 3.63x - 3.88$  \ ($R^2 = 0.9739$)</td>
</tr>
<tr>
<td>High</td>
<td>$Y = 3.43x + 0.18$  \ ($R^2 = 0.9990$)</td>
<td>$Y = 3.18x + 0.42$  \ ($R^2 = 1$)</td>
<td>$Y = 5.18x + 3.27$  \ ($R^2 = 0.999$)</td>
<td>$Y = 4.02x - 7.37$  \ ($R^2 = 0.9986$)</td>
</tr>
</tbody>
</table>

Table 6: Analytical figures of merit used for the quantification of adrenergic amines

The reproducibility of both the retention time and the peak area of each amine were studied, as illustrated in table 7. With regards to average retention time, there was very little variability. The relative standard deviation of tyramine was much higher with chemiluminescence detection when compared to the other amines, but still well within the limits of acceptable variance. The average peak area of the amines was also found to be reproducible.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative standard deviation (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. Peak area</td>
</tr>
<tr>
<td></td>
<td>CL</td>
</tr>
<tr>
<td>Synephrine</td>
<td>2.20</td>
</tr>
<tr>
<td>Octopamine</td>
<td>1.72</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0.54</td>
</tr>
<tr>
<td>Hordenine</td>
<td>2.91</td>
</tr>
</tbody>
</table>

Table 7: Reproducibility data for peak area and retention time of each adrenergic amine with HPLC coupled to ultraviolet (UV) absorbance and chemiluminescence (CL) detection.

Information supplied on weight-loss product labels can include the equivalent mass of dried *C. aurantium* fruit, the actual quantity of dried extract added to the product, or the specific mass of synephrine. However, significant differences between the claimed and measured quantities of ingredients have been reported for some of the weight-loss products containing ephedra alkaloids and/or synephrine that were available prior to the FDA’s restriction on ephedrine\textsuperscript{90,91}. Standardised alcohol and water extracts (ranging from 3\% to 95\% synephrine) from the immature fruit are produced as ingredients for dietary supplements. Analysis of two standardised extracts shows the quantities of synephrine, established using HPLC with chemiluminescence detection were in good agreement with those obtained using HPLC with UV-absorbance detection.
(n= 5, > 2% RSD) and above the minimum values stated by the manufacturer (table 8).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quantity of Synephrine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Claimed</td>
</tr>
<tr>
<td>Extract A</td>
<td>≥ 6</td>
</tr>
<tr>
<td>Extract B</td>
<td>≥ 10</td>
</tr>
</tbody>
</table>

**Table 8: Quantity of synephrine in standardised *C. aurantium* extracts**

The monolithic column HPLC methodology developed in this chapter, using UV-absorbance and chemiluminescence detection, was also applied to the determination of synephrine in currently available dietary supplements that contain *C. aurantium* extracts in addition to extracts from other herbs (labelled as products 1-4). The two methods of detection gave similar results for the determination of synephrine in these dietary supplements (Table 9). The quantity of synephrine varied from 0.8% to 6.2% (% m/m) (6.7 – 44.7 mg synephrine per tablet or capsule), which falls within the wide range reported by Avula et al. (0.07 – 18.6%) for eight dietary supplements containing *C. aurantium* extracts.

The quantity of extract added to each of the four products was reported on the labels as an equivalent weight of dried *C. aurantium* fruit. The percentage mass
of synephrine in the specified quantities of dried fruit was calculated to be between 0.22% and 0.38% (% m/m, Table 9), which is within the range reported by Avula et al. for 13 dried samples of unripe and ripe fruits of *C. aurantium* (0.04 – 0.41%), and similar to the value reported by Pellati et al. for one sample of dried fruit (0.35%).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quantity of Synephrine (%m/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In tablet / capsule</td>
</tr>
<tr>
<td></td>
<td>HPLC - UV</td>
</tr>
<tr>
<td>Product 1</td>
<td>5.6</td>
</tr>
<tr>
<td>Product 2</td>
<td>0.89</td>
</tr>
<tr>
<td>Product 3</td>
<td>1.3</td>
</tr>
<tr>
<td>Product 4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**Table 9:** Quantity of synephrine in dietary supplements containing *C. aurantium* extracts

During this study another dietary supplement (referred to as ‘Product 5’) was obtained which contained (as claimed on the label) a standardized *C. aurantium* extract (95% synephrine) and specified amounts of pure compounds, including octopamine, tyramine and hordenine (table 10). Two modes of detection (UV
absorbance and chemiluminescence) were applied for the quantification of the amines.

<table>
<thead>
<tr>
<th>Product 5 Compounds</th>
<th>Quantity per capsule (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Claimed</td>
</tr>
<tr>
<td>Octopamine</td>
<td>10.7</td>
</tr>
<tr>
<td>Syneprine</td>
<td>3.4</td>
</tr>
<tr>
<td>Tyramine</td>
<td>5.3</td>
</tr>
<tr>
<td>Hordenine</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*Table 10*: Comparison of the claimed and measured amount of adrenergic amines in product 5.

The quantity of hordenine, detected with chemiluminescence and UV-absorbance, was in relatively good agreement with the claimed amount. Syneprine was determined to be 35% higher than the stated amount with both modes of detection.

The determined amount and the claimed amount of octopamine differed by 17%, using UV-absorbance detection, however the amount determined by chemiluminescence detection was much closer to the specified value. With this product only the octopamine peak was observed with chemiluminescence detection, however the UV chromatogram detected two compounds co-
eluting. This may have resulted in the inconsistency observed for the amount of octopamine in product 5.

Information supplied for the product also indicated that a relatively substantial amount of tyramine, as a pure compound, had been added to the nutraceutical. The claimed amount was 50 mg (which equates to 5.3% (m/m) per capsule), however no tyramine was detected in this product. The product was re-examined using different extraction solutions (250 mL water; 50 mL 0.1 M HCl; 50 mL of 70% ethanol in water), but tyramine was still not detected. The product was then spiked with 50 mg of tyramine, extracted with the above solutions and re-quantified. In all cases a near 100% recovery of tyramine was achieved with the spiked sample.
4. Conclusion

Adrenergic amines were found to elicit light as a result of their reaction with acidic potassium permanganate, however an interference study suggested that separation of these amines in weight-loss supplements would be necessary before quantification. A fast, reproducible method for the determination of adrenergic amines in dietary supplements was developed, based on a monolithic column separation and chemiluminescence detection. The amines were quantified in five weight-loss supplements and were found to be between 0.5% and 7%, which is within the range previously reported. Chemiluminescence detection provided greater selectivity than UV-absorbance, which is particularly important in samples that include other herbal extracts and many potentially interfering species. The work presented in this chapter has been published in the literature^{12,100}. 
Chapter 3

Manganese(II) enhanced acidic potassium permanganate chemiluminescence

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4. Conclusion
1. Introduction

There are currently numerous analytical applications of acidic potassium permanganate chemiluminescence, as described in two comprehensive reviews on the subject\textsuperscript{37,38}. The mechanism for this reaction is discussed in greater detail in chapter 4.

Selectivity associated with chemiluminescence is a product of only a small number of chemical reactions of this type yielding significant amounts of light. Light-producing redox reactions involving acidic potassium permanganate usually have the same emitting species, regardless of the analyte\textsuperscript{115}, thus prohibiting wavelength differentiation. Due to these conditions chemiluminescence detection often requires coupling with highly selective analytical tools, such as chromatography, however such pairings achieve a sensitive and interference-free measurement\textsuperscript{1}.

Light emitted from a chemical reaction with acidic potassium permanganate can be improved by enhancers; the two most commonly used, formaldehyde\textsuperscript{41,61,116-122} and sodium polyphosphate\textsuperscript{11,39,40,50,123-128}, both provide significant increases in emission intensity.

Highly fluorescent compounds, such as Rhodamine B, Rhodamine 6G and quinine, have been tested as potential enhancers of the potassium permanganate chemiluminescence reaction\textsuperscript{129-131}. While enhancement from these compounds has been observed in some cases, it has been limited to a relatively small number of applications.
Formaldehyde has been found to be a good enhancer in many studies\textsuperscript{41,61,116-122}, with reports of an increase of up to two orders of magnitude in some cases\textsuperscript{47,132,133}. Unfortunately formaldehyde reacts with permanganate to produce a blank signal, which sometimes negates the observed enhancement\textsuperscript{134}. Additionally, formaldehyde is a known carcinogen\textsuperscript{135}, therefore the use of this compound is undesirable, and requires special precautions when being handled. Other aldehydes have also been tried, and while some have been found to increase the chemiluminescence response of certain analytes, the enhancement is often negated by the production of a blank signal, arising from the reaction of the aldehydes with the reagent, or in other cases, the alternate aldehydes have been reported to be inferior to formaldehyde\textsuperscript{41,46,47,61,116,119,121,133,136}.

Various polyphosphates increase the chemiluminescence intensity of permanganate reactions, including sodium polyphosphate, sodium hexametaphosphate, sodium dihydrogen orthophosphate, and several phosphoric acids\textsuperscript{11,39,40,50,123-128}. Significant increases in intensity have been observed from these enhancers. Abbott\textsuperscript{36} and co-workers concluded that the stability of the manganese complex seems to increase with longer polyphosphate chains. Additionally, it has been noted that the presence of polyphosphates shifts the emission maximum from 734 ± 5 nm to 698 ± 5 nm\textsuperscript{50}. The enhancement mechanism, as proposed by Hindson\textsuperscript{137} et al. involves sodium polyphosphate improving the permanganate chemiluminescence intensity by preventing the formation of insoluble manganese(IV) species, thus stabilising manganese(III), and forming a protective “cage-like” structure around the
excited-state manganese(II)* emitter, thereby inhibiting non-radiative relaxation. Other polyphosphates are also thought to enhance the reaction by stabilisation of the reaction intermediates.

Metal ions, including copper, iron and manganese ions, have also been investigated as potential enhancers of the permanganate reaction\textsuperscript{138,139,140}. Tsaplev\textsuperscript{141} generated manganese(II) by reducing potassium permanganate with ethanol, and noted a six-fold increase in the emission intensity of the reagent with morphine. He concluded that the chemiluminescence intensity is dependent on manganese(II) concentration. Zhu\textsuperscript{45} and colleagues noted a considerable increase in the oxidation of butane-2,3-dione by potassium permanganate when manganese(II) was added. They utilised the increase in the kinetics of this reaction to determine the concentration of the metal ion.

In this chapter, work is presented which leads to the conclusion that manganese(II) can enhance acidic potassium permanganate chemiluminescence by up to two orders of magnitude. The manganese(II) salt is used in addition to sodium polyphosphate as enhancers of the reaction. The work presented in this chapter has been published in the literature\textsuperscript{142-144}. 
2. Experimental

2.1 Reagents, standards and samples.

Unless otherwise stated, the standard acidic potassium permanganate reagent \( (1 \times 10^{-3} \text{ M}) \) was prepared by dissolving potassium permanganate (Chem-Supply, Gillman, SA, Australia) in a 1\% (w/v) sodium polyphosphate (+80 mesh; Sigma-Aldrich, Castle Hill, NSW, Australia) solution and adjusted to pH 2.5 with sulphuric acid. The enhanced permanganate reagent consisted of preparing a solution of standard acidic potassium permanganate (1 mM, 1\% w/v sodium polyphosphate, pH 2.5) and adding to it the desired amount of manganese(II) acetate or manganese(II) sulphate, as indicated. This reagent was prepared and stored for 24 hours, to ensure the solution was at equilibrium, prior to analysis. An alternative enhanced reagent was later developed, which did not require the lengthy sitting period. This reagent was prepared by reducing acidic potassium permanganate (1.9 mM, 1\% w/v sodium polyphosphate, pH 2.5) with sodium thiosulphate (0.6 mM).

2.2 Instrumentation

Flow injection analysis manifold was constructed as described in chapter 2 of this thesis, except that the volume of the sample loop was 70 \( \mu \text{L} \). The chemiluminescence signal was collected using an ‘e-corder 410’ data acquisition system (eDAQ, Denistone East, NSW, Australia).
High performance liquid chromatography (HPLC) separation was performed using a HP1100 liquid chromatography system (Agilent Technologies, Forest Hill, Victoria, Australia) with a Chromolith SpeedROD column (RP-18 endcapped, 50 mm length × 4.6 mm i.d.) and 5 mm monolithic guard column (Merck, Kilsyth, Victoria, Australia). The system was configured as previously described\textsuperscript{145,146}, except that a GloCel detector with single-inlet serpentine-channel reactor\textsuperscript{147} was used. The column eluent merged with the permanganate reagent at a T-fitting immediately prior to entering the detector. The injection volume was 20 μL.

UV-Visible absorption spectra were obtained using a Cary 300 Bio UV-visible spectrophotometer (Varian, Mulgrave, Australia) with 10 mm pathlength, sealable quartz cuvettes (Starna, Baulkham Hills BC, NSW, Australia).

Stopped-flow experiments were performed with a flow injection analysis manifold consisting of a programmable dual syringe pump (Model sp210iw, World Precision Instruments, Glen Waverly, Victoria, Australia), Valco six-port injection valve (SGE, Ringwood, Victoria, Australia), and GloCel chemiluminescence detector with dual-inlet serpentine-channel reaction zone\textsuperscript{147} (Global FIA), which allowed the merging of solutions directly in front of the photomultiplier (Electron Tubes model P30A-05; ETP, Ermington, NSW, Australia) and therefore the entire chemiluminescence intensity versus time profile was captured. The serpentine reaction zone has previously been shown to enhance mixing efficiency compared to the conventional spiral configuration\textsuperscript{147,148}. 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. The output signal from the photomultiplier was recorded using an ‘e-corder 410’ data acquisition (eDAQ, Denistone East, NSW, Australia).
3. Results and Discussion

3.1 Initial findings

The stability of acidic potassium permanganate solutions is dependent on both the degradation of the reagent\textsuperscript{149} and the hydrolysis of the polyphosphates\textsuperscript{150}. While the hydrolysis of the polyphosphates is time and pH dependant, the degradation of the oxidant is thought to occur faster in the presence of light.

Two solutions of acidic potassium permanganate (1 mM, 1% w/v sodium polyphosphate crystals, adjusted to pH 2.5 with sulfuric acid) were prepared. One of the solutions was protected from light by covering in aluminum foil and storing in the dark, whereas the other solution was left exposed to natural light. In doing so, the only difference between the two solutions was the storage conditions. The two solutions are herein after referred to as “Dark” and “Light” permanganate, in reference to their storage conditions. The solutions were left standing for six months, to determine the effects of polyphosphate hydrolysis and oxidant degradation on the chemiluminescent signal intensity.

Comparison of a freshly prepared permanganate solution (1 mM, 1% w/v sodium polyphosphates, pH 2.5 adjusted with sulphuric acid) with the two 6-month old permanganate solutions revealed unexpected differences in chemiluminescence intensity (Figure 13).
Figure 13: Chemiluminescence signal comparison of a fresh permanganate solution with six month old solutions, one protected from light ("Dark"), and one exposed to natural light ("Light").

Four analytes were selected for comparison – tyramine, octopamine, 3-aminophenol and 2,3-diaminophenol. The signal intensity of each of the analytes, measured as peak height, upon oxidation with a freshly prepared potassium permanganate reagent was compared to the signal intensity of the four analytes upon reaction with "Dark" permanganate. As expected, the six month old "Dark" solution showed a significant reduction in signal intensity (an approximate 10 fold decrease). This result was anticipated as, over time, both
the hydrolysis of polyphosphates and the degradation of oxidant are expected to reduce the emission maximum generated by the reagent.

The unexpected results were obtained when the four analytes were reacted with “Light” permanganate, a six month old permanganate solution which had been exposed to natural light. As shown in figure 13, the signal obtained with this permanganate solution was higher than the signal obtained with a fresh permanganate solution in the case of tyramine, octopamine and 3-aminophenol, but not with 2,3-diaminophenol. Based on this observation, an investigation was undertaken to understand what changes had occurred within the “Light” permanganate that would explain the increased intensity of three out of the four analytes examined (figure 13).

The pH of a fresh solution is adjusted to 2.5 by drop-wise addition of sulfuric acid. The pH of each of the two 6-month old solutions was found to be 2.67 for “Dark” and 2.73 for “Light”. While there is a slight increase from the initially adjusted pH 2.5, the difference is not likely to be significant enough to cause such deviations in the chemiluminescence signal shown in figure 13.

Manganese(II), a byproduct of the degradation of manganese(VII), has previously been shown to effect certain permanganate reactions. Several researchers have described a change in permanganate chemiluminescence intensity or reaction rate in the presence of manganese(II) salts\textsuperscript{118,151-154}. Agater\textsuperscript{155} and co-workers added 200 mM manganese(II) to accelerate the very slow chemiluminescent oxidation of carbohydrates, to obtain limits of detection of 1×10^{-4} M. As mentioned in the introduction to this chapter, Zhu\textsuperscript{152} and co-workers
determined manganese(II) based on its influence on the rate of the reaction between permanganate and 2,3-butanedione. Townshend\textsuperscript{153,154,156} and colleagues have previously added manganese(II) to permanganate to enhance the chemiluminescence. Their setup involved an on-line merger of manganese(II), the analyte and the reagent (which did not contain polyphosphates) prior to analysis, where a 16\% enhancement was observed for one analyte\textsuperscript{156}, and a 51\% and 80\% decrease was observed with two other analytes\textsuperscript{153,154}. However, at that time, the emitting species in these reactions had not yet been confirmed\textsuperscript{157,158} (the luminescence was often erroneously attributed to the production of singlet oxygen\textsuperscript{118,159}) and the full implications of these observations were not apparent.

The disparity in the chemiluminescent signal observed with the “Dark” and “Light” reagents is hypothesized to be caused by the presence of manganese(II), formed by the reduction of manganese(VII) when exposed to light. It is proposed that manganese(II) is further enhancing the chemiluminescent reaction, as observed with the “Light” reagent, thus counteracting the undesired effects caused by the slow hydrolysis of sodium polyphosphates.

### 3.2 Spectroscopic studies

Further investigation was undertaken employing ultraviolet-visible (UV-visible) spectroscopy. A typical UV-visible absorbance spectrum (700 – 250 nm) of potassium permanganate is shown in figure 14. The permanganate solution has a unique double peak in the 450 – 600 nm range. Maximum absorbance is
observed at 525 nm, with other notable peaks at 545 nm, and at 310 nm (figure 14).

![Absorbance vs Wavelength](image)

**Figure 14:** Typical ultraviolet-visible scan of 1mM potassium permanganate, 1% m/v sodium polyphosphates, adjusted to pH 2.5 with sulphuric acid.

Based on spectrophotometric calibrations at 525 nm (shown in figure 15), the concentration of potassium permanganate in the “Dark” reagent was calculated to be 5.7% lower than that of a fresh permanganate reagent, whereas the concentration of the six month old “Light” permanganate solution had decreased by 29.5%. This result indicates that exposure to light increases the degradation of the manganese(VII) reagent.
Figure 15: Calibrations of freshly prepared acidic potassium permanganate and the two 6-month-old permanganate solutions, one exposed to natural light and one exposed to no light. The absorbance was measured at 525 nm.

To artificially replicate the six month old, light exposed permanganate reagent, a new reagent was made under following conditions: 0.7 mM potassium permanganate, 1% w/v sodium polyphosphate, adjusted to pH 2.5 with sulfuric acid and finally 0.3 mM manganese(II) acetate was added. The manganese salt was added after the sodium polyphosphate and the pH had been adjusted. These parameters were initially selected due to the spectroscopic results. A 1 mM solution of potassium permanganate had approximately 30% less potassium
permanganate after being exposed to light, and it was assumed that this permanganate was converted to manganese(II), thus a reagent containing 0.7 mM permanganate and 0.3 mM of manganese(II) was prepared.

A quick screening, using tyramine and adrenalone as analytes, revealed that a higher chemiluminescence intensity was observed with the permanganate/manganese(II) reagent than that of the "Light" or fresh permanganate reagents (figure 16).

![Graph showing chemiluminescence comparison](image)

**Figure 16**: Comparison of freshly prepared standard potassium permanganate, "Dark" permanganate, "Light" permanganate and permanganate/manganese(II) acetate (0.7 mM / 0.3 mM) reagents. The samples were tyramine ($5 \times 10^{-6}$ M) and adrenalone ($1 \times 10^{-4}$ M).
Furthermore, it was noted that re-addition of fresh 1% w/v sodium polyphosphates to the two 6-month old permanganate solutions increased the chemiluminescence signal intensity of tyramine by 2 – 5 fold (figure 17).

The data shown in figures 16 and 17 indicate that (i) a significant portion of the intensity loss observed with the “Dark” permanganate reagent is due to the hydrolysis of the polyphosphates, and (ii) adding manganese(II) to potassium permanganate enhances the chemiluminescence intensity observed with that reagent.

Figure 17: Signal comparison of 6-month old “Light” and “Dark” reagents with and without adding fresh polyphosphates (1% w/v). The analyte is tyramine (1 x 10^{-5} M).
3.3 Optimization of manganese(II) concentration

In the initial investigations, manganese(II) was added to the permanganate reagent in the form of manganese(II) acetate. This source of manganese(II) was then changed to manganese(II) sulfate, to understand if there was any impact on the chemiluminescence signal caused by the acetate in the matrix. Comparison of the signal enhancement when manganese(II) acetate (0.3 mM) and manganese(II) sulfate (0.3 mM) are added to a freshly prepared permanganate solution (figure 18) shows little difference between the two sources of manganese(II). While manganese(II) acetate yielded a slightly higher signal, the overall enhancement observed with both manganese(II) enhancers was of a similar magnitude and significantly higher than the signal obtained from permanganate without the manganese(II) added.
Figure 18: Chemiluminescent enhancement observed from Mn(II) acetate (0.3 mM) and Mn(II) sulphate (0.3 mM) added to standard potassium permanganate (1 mM, 1% w/v sodium polyphosphate, pH 2.5, adjusted with sulphuric acid). All analytes were $1 \times 10^{-5}$ M.

Originally, based on the UV-visible results of the 6-month old permanganate solutions, 0.3 mM manganese(II) acetate was added to a permanganate solution to yield the above observed enhancement in chemiluminescence. A study to determine the optimum concentration of the metal ion enhancer was subsequently undertaken. Initial results (figure 19) show that the optimum manganese(II) concentration is 0.3 mM manganese(II) for tyramine and octapamine.
Figure 19: Signal intensity of octapamine and tyramine ($1 \times 10^{-5}$ M) relative to concentration of manganese(II) added to potassium permanganate (1 mM, 1% w/v polyphosphate, pH 2.5).

Several other compounds were analyzed with flow injection analysis (figure 20), using manifold 3, shown in figure 6, and show the optimum enhancer concentration as either 0.3 mM or 0.6 mM. Maximum chemiluminescence emission was found to be dependent on both the analyte and the instrumental approach, but in all cases examined was found to be 0.3 - 0.6 mM manganese(II).
During the manganese(II) optimisation tyramine was determined twice, in the morning and evening of the same day. The duplicates were in good agreement. Interestingly the signal observed for tyramine during this study (approximately 7000 mV, figure 20) was much higher than during previous studies (approximately 2500 mV, illustrated in figures 17 and 18, and 4000 mV as shown in figure 19), at the same concentration. In the preceding work tyramine was determined immediately after the preparation of the permanganate/manganese(II) reagent, whereas during this study, the reagent
was prepared in the evening and the analysis occurred the following morning. On this basis, it was thought that once the manganese(II) is added to the permanganate reagent, some time is required for the solution to reach equilibrium before the maximum chemiluminescence signal can be obtained.

3.4 Solution equilibrium

It is important to understand the behavior of the permanganate/manganese(II) reagent over the day, to ensure it’s stability and usefulness as a chemiluminescence reagent. A potassium permanganate/manganese(II) acetate reagent was prepared (1 mM KMnO₄ / 0.3 mM manganese(II) acetate) as follows; first the 1 mM permanganate solution was prepared, to which was added 1% w/v sodium polyphosphate, and the pH adjusted to 2.5 with sulphuric acid, and finally the manganese(II) salt was added to the solution. The addition of manganese(II) to potassium permanganate before sodium polyphosphate results in the formation of a precipitate, and visually the colour of the solution changes from a dark purple/pink, characteristic of potassium permanganate, to a deep red colour. Hindson et al. have shown that the polyphosphates prevent the formation of insoluble manganese(IV) oxides, therefore in preparation of this reagent sodium polyphosphate must be added before the addition of manganese salts to the solution.

A permanganate/manganese(II) acetate reagent was prepared and left standing overnight. A duplicate reagent was prepared fresh in the morning, and the
phosphorescence from the reduction of this reagent by tyramine and octopamine was measured (octopamine results shown in figure 21).

Figure 21: Hourly analysis of octopamine (1 x 10^-5 M) with two permanganate/manganese(II) reagents (1 mM / 0.3 mM), one prepared just prior to analysis and one prepared the night before.

The chemiluminescence response obtained with the overnight reagent was much higher than that of the freshly prepared permanganate/manganese(II) reagent (figure 21), and the response from this reagent remained steady over
the duration of the experiment. On the other hand, the signal observed from the freshly prepared reagent steadily increased every hour, over the period of analysis. This trend was observed with both tyramine and octopamine (figure 22), and confirmed that a substantial amount of time is required for the permanganate and manganese(II) mixture to reach equilibrium, before the maximum response in observed. Additional experiments showed an equilibration period of approximately 12 hours before the plateau is reached (see figure 22). After this point the emission intensity shows minimal variation over the remaining 24 hour period, and was later shown to be stable over 48 hours, which was the maximum time tested.
Figure 22: Chemiluminescence signal over time profile for tyramine and octopamine \((1 \times 10^{-5} \text{ M})\) with freshly prepared potassium permanganate/manganese(II) acetate reagent.

The chemiluminescence response over time for octopamine (figure 22) shows a relatively constant increase in signal intensity until the roll-over point is reached (approximately 12 hours), than the signal remains comparatively stable thereafter. Tyramine, however, is more irregular in its chemiluminescence response over the 24 hours. The experiment was repeated several times and tyramine’s response maintained a somewhat unpredictable behavior, however the general shape remained similar to that of octopamine.
As mentioned earlier, Townshend\textsuperscript{156} and co-workers noted a 16% increase in chemiluminescence for one analyte when they added 1 mM manganese(II) to the carrier stream, but a decreased signal of 50% - 80% for two other analytes\textsuperscript{153,154}. The manganese(II) was added online, therefore it is likely that insufficient time was allowed for the manganese(II) to equilibrate with the permanganate, resulting in the relatively low signal enhancement observed.

### 3.5 Reagent stability

Acidic potassium permanganate solutions containing sodium polyphosphate have limited stability due to both the reduction of the oxidant\textsuperscript{149} and a pH dependent hydrolysis of the polyphosphate\textsuperscript{160}. Solutions that provide the optimum conditions for chemiluminescence detection (e.g. 1 mM KMnO$_4$, 1% w/v sodium polyphosphate, adjusted to pH 2.5 with H$_2$SO$_4$) are sufficiently stable for most applications, but show some variation over 48 hours. Accordingly the feasibility of the permanganate/manganese(II) reagent is dependent on the stability of this reagent.

Ultraviolet-visible absorption spectroscopy was used to examine the stability of the permanganate/manganese(II) reagent by measuring changes in absorbance of the enhanced permanganate reagent (using manganese(II) acetate and manganese(II) sulphate) and comparing to the absorbance values of standard acidic potassium permanganate. A scan (700 – 250 nm) for each reagent was attained every 30 minutes for a period of 48 hours. The regions of the spectrum of particular interest are at 525 nm ($\lambda_{\text{max}}$ for potassium permanganate) and at
310 nm. Increase in absorbance at the latter wavelength can be attributed to the formation of soluble manganese(III) in solution\(^{137}\).

The standard potassium permanganate reagent shows almost no change in absorbance (at 525 nm and 310 nm) over 48 hours (figure 23), with a 1.3% difference between the highest and lowest absorbance values at both wavelengths.

Figure 23: Change in UV-visible absorbance of acidic potassium permanganate at 525 nm and 310 nm over 48 hours.
However potassium permanganate with manganese(II) acetate (figure 24) shows a 4.9% decrease in absorbance at 525 nm over the first 12 hours. Manganese(II) sulfate shows a very similar movement to that shown in figure 24, with a 5.6% decrease in absorbance at the same wavelength. Additionally, both manganese(II) acetate and manganese(II) sulphate show an increase in absorbance, at 310 nm, of 8.7% and 7.0%, respectively.

Figure 24: Change in UV-visible absorbance of acidic potassium permanganate/manganese(II) acetate (1 mM KMnO₄ / 0.3 mM Mn(II)CH₃COOH) at 525 nm and 310 nm over 48 hours.
The increase in the absorbance at 310 nm, as noted with both permanganate/manganese(II) reagents, also takes approximately 12 hours to plateau. After this 12 hour period, there is very little variation in absorbance at both wavelengths, and with both reagents, over the remaining 36 hours examined. Coincidently, the time it takes for the permanganate/manganese(II) absorbance to stabilize is approximately similar to the time required for this reagent to reach equilibrium (discussed in section 3.3 of this chapter). The change in chemiluminescence intensity mirrored the slow oxidation of manganese(II) by permanganate (measured as a decrease in absorbance at 525 nm), as illustrated in figure 24. A corresponding rise in absorbance at 310 nm (figure 23) indicated the formation of soluble manganese(III) species\textsuperscript{161}. 

**Figure 25:** Increase in chemiluminescence intensity (with octopamine) for a reagent containing $1 \times 10^{-3}$ M potassium permanganate and $3 \times 10^{-8}$ M manganese(II) sulfate in 1% m/v sodium polyphosphate, adjusted to pH 2.5 with sulfuric acid, and the decrease in reagent absorbance at 525 nm.

A chemiluminescence experiment with tyramine, using sequential injection analysis, resembled the results obtained with octopamine (figure 25), and also showed that the reagent was stable for 48 hours, after the initial equilibration period of approximately 12 hours$^{142}$. A comparison of the results, employing UV-visible data, obtained for permanganate/manganese(II) sulphate also shows a clear correlation between the decrease in the absorbance (at 525 nm) over 12 hours and an increase in the chemiluminescence signal over the same time
frame. The data indicates an interaction between the manganese(VII) species in potassium permanganate and the manganese(II) species (as observed with acetate and sulphate salts). The two reagents, once mixed, both reach equilibrium in about 12 hours, and remain stable for 48 hours, which was the maximum time tested. From this point forward the permanganate/manganese(II) reagent was prepared one day prior to analysis, to insure the solution equilibrated and the maximum intensity was recorded.

3.6 Kinetic analysis

Kinetic studies were commenced by obtaining chemiluminescence intensity versus time profiles for numerous organic compounds. The oxidation of adrenergic amines (octapamine, synephrine, tyramine and hordenine) by acidic potassium permanganate was found to be very slow (figure 26). Maximum emission for these analytes occurred between 10 and 30 seconds after the analyte and reagent were merged. Synephrine, the slowest of the four adrenergic amines examined, was still reacting with the reagent 80 seconds after the initial mixing. While these amines have been shown to respond well with the permanganate reagent (discussed in chapter 2), their kinetic profile, shown in figure 26, indicates flow analysis detection is not the ideal method for their determination.
Figure 26: Kinetic profile for the oxidation of adrenergic amines by acidic potassium permanganate (1 mM, 1% w/v polyphosphates, pH 2.5 adjusted with H$_2$SO$_4$). All analytes were 1 x 10$^{-5}$ M.

Addition of 0.3 mM manganese(II) acetate to potassium permanganate resulted in a considerable increase in the rate of oxidation of adrenergic amines by the reagent, as shown in figure 27. The emission maxima were observed within 3 seconds with all four amines, as opposed to 10 - 30 seconds when manganese(II) is not added to the permanganate reagent. Earlier data showed that manganase(II), when added to permanganate, resulted in a significant enhancement of the chemiluminescence signal of tyramine (discussed in section 3.1 of this chapter). Taking into consideration the results summarized in table 11,
it is evident that manganese(II) drastically increases the rate of reaction of tyramine, and other adrenergic amines, with potassium permanganate, thereby making the analytes much more suitable to flow analysis detection. As such, these analytes yielded a much more intense emission with this reagent.

Figure 27: Chemiluminescence response over time for the oxidation of adrenergic amines by acidic potassium permanganate/manganese(II) acetate (1 mM permanganate, 1% w/v polyphosphates, pH 2.5 adjusted with H₂SO₄ and 0.3 mM manganese acetate). All analytes were 1 x 10⁻⁵ M.
<table>
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<th>Potassium permanganate with manganese(II)</th>
<th>Synephrine</th>
<th>Octopamine</th>
<th>Tyramine</th>
<th>Hordenine</th>
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<td>1.4</td>
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</tr>
</tbody>
</table>

**Table 11:** Time taken to reach maximum emission. All analytes were $1 \times 10^{-5}$ M.

The increase in the reaction rate of adrenergic amines with potassium permanganate is directly proportional to the amount of manganese(II) added to the reagent (table 11). With synephrine, for example, the time required to reach maximum emission decreases by a factor of 6 when 0.05 mM manganese(II) is added to the permanganate reagent. Furthermore, under the same conditions, a 5-fold increase in the signal intensity of synephrine is noted (Figure 28). Both the reaction rate and intensity continue to increase with further additions of manganese(II). Interestingly, when 0.6 mM manganese(II) is added, the reaction kinetics are further enhanced, but the chemiluminescence intensity decreases, compared to 0.3 mM manganese(II).

Recent work by Terry et al. has demonstrated that the design of flow-cells can have a large influence on the chemiluminescence signal with flow injection analysis. The flow cell used in these experiments was a single inlet, spiral cell,
which means that the reagent and analyte are mixed prior to entering the cell coil for detection. Decrease in the chemiluminescence intensity of synephrine with the 0.6 mM manganese(II) reagent may be attributed to the loss of light prior to the mixture entering the flow cell, due to the now very fast rate of oxidation of synephrine.

![Graph showing chemiluminescence intensity over time](image)

**Figure 28:** Reaction rate of synephrine ($1 \times 10^{-5}$ M) with acidic potassium permanganate and $0 - 0.6$ mM manganese(II) acetate.
Morphine, on the other hand, is very quickly oxidized by permanganate (figure 29). Even so, the addition of manganese(II) to permanganate further increases the rate of morphine oxidation. The emission maximum with standard potassium permanganate reagent (reached at 1.5 seconds) is reduced to 0.9 seconds with the 0.6 mM manganese(II) reagent. The overall trend demonstrated an increase in both the intensity and reaction kinetics with increasing amounts of manganese(II), with 0.3 mM manganese(II) permanganate reagent elucidating the largest chemiluminescence response, and 0.6 mM manganese(II) resulting in the fastest reaction rate. Once again the decrease in the signal intensity with 0.6 mM manganese(II) reagent is likely due to the design of the instrumentation. Utilizing a double inlet cell, where the reagent and analyte streams are merged inside the flow cell, a greater signal is achieved with very fast reactions.\textsuperscript{143}
**Figure 29**: Reaction rate of morphine with potassium permanganate and increasing amounts of manganese(II).

The kinetic profiles examined above show the potential advantage in having control of reaction rates, particularly in flow systems. Additionally, it is evident that the design of flow cells can have a significant impact on instrumental capabilities with flow injection analysis. To illustrate this point, luminous oxidations of syneprhine and morphine were photographed (figure 30).

A photograph of the reaction of syneprhine with potassium permanganate (figure 30.a) shows a dull red glow, which is increasing in intensity on the outer edges of the flow cell, as the mixture is exiting the cell. This visually confirms that
syneprine is still reacting with acidic potassium permanganate as the solution exits the flow cell. As 0.3 mM manganese(II) is added to the permanganate reagent (figure 30.b), the reaction kinetics are considerably increased, and the most intense light emission is observed in the centre of the flow cell. The intensity dies down towards the outer edges of the cell. As most of the reaction is now occurring within the flow cell, and is therefore being detected by the photomultiplier tube, a significant chemiluminescence signal enhancement for syneprine is observed. Adding more manganese(II) (0.6 mM, figure 30.c) further increases the reaction rate. The increase in the reaction kinetics is also noted with morphine (figures 30 d,e,f).
Figure 30. Photographs of chemiluminescence from the single-inlet serpentine flow cell of the GloCel detector. Parts (a), (b), and (c) show the reaction between synephrine and acidic potassium permanganate, with 0 mM, 0.3 mM, and 0.6 mM manganese(II) sulfate, respectively, using an exposure time of 5 min. Parts (d), (e), and (f) show the reaction of morphine with the same three reagent conditions and an exposure time of 2.5 s. Each reagent contained 1% m/v sodium polyphosphate and was adjusted to pH 2.5 with sulfuric acid prior to adding manganese(II). The reagent and analyte (1 × 10^{-3} M) were continuously merged at a T-piece immediately prior to entering the detector.

3.7 Applications of the new reagent

Four important classes of analytes – antioxidants, adrenergic amines, opiate alkaloids and neurotransmitters and metabolites – that have previously been detected with potassium permanganate have been re-examined to investigate the potential of manganese(II) acetate as an enhancer of the chemiluminescence reactions between the above mentioned analytes and potassium permanganate.

The results are summarized in table 12 below. The chemiluminescence enhancement was observed to be the greatest with adrenergic amines, with over a 100 fold intensity enhancement observed for synephrine and octopamine. Tyramine and hordenine signals were enhanced by approximately 50 times. This enhancement can primarily be attributed to increasing the otherwise very slow oxidation of these amines, thus increasing the reaction rate and making these compounds much more suited to flow analysis detection.

Vanilmandelic acid and homovanillic acid were enhanced 15 and 7 fold, respectively, but interestingly the other neurotransmitter metabolites
(dopamine and serotonin) showed practically no enhancement. Quercetin, an antioxidant, also showed no enhancement with the manganese(II) reagent. Despite this, the data in table 12 indicates that using manganese(II) with potassium permanganate has a great potential to significantly enhance the chemiluminescence of numerous organic compounds.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>KMnO$_4$</th>
<th>KMnO$_4$ + Mn(II)</th>
<th>Enhancement factor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neurotransmitters and metabolites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dopamine</td>
<td>720</td>
<td>750</td>
<td>1</td>
</tr>
<tr>
<td>vanilmandelic acid</td>
<td>240</td>
<td>3580</td>
<td>15</td>
</tr>
<tr>
<td>homovanillic acid</td>
<td>385</td>
<td>2610</td>
<td>7</td>
</tr>
<tr>
<td>serotonin</td>
<td>8570</td>
<td>10200</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Adrenergic amines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>octopamine</td>
<td>90</td>
<td>9400</td>
<td>103</td>
</tr>
<tr>
<td>synephrine</td>
<td>55</td>
<td>6400</td>
<td>113</td>
</tr>
<tr>
<td>tyramine</td>
<td>170</td>
<td>7780</td>
<td>46</td>
</tr>
<tr>
<td>hordenine</td>
<td>130</td>
<td>9000</td>
<td>68</td>
</tr>
<tr>
<td><strong>Opiate alkaloids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>morphine</td>
<td>28100</td>
<td>170300</td>
<td>6</td>
</tr>
<tr>
<td>codeine</td>
<td>70</td>
<td>227</td>
<td>3</td>
</tr>
<tr>
<td>oripavine</td>
<td>14170</td>
<td>137870</td>
<td>10</td>
</tr>
<tr>
<td>pseudomorphine</td>
<td>22160</td>
<td>48400</td>
<td>2</td>
</tr>
<tr>
<td><strong>Antioxidants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>resveratrol</td>
<td>130</td>
<td>510</td>
<td>4</td>
</tr>
<tr>
<td>quercetin</td>
<td>664</td>
<td>654</td>
<td>1</td>
</tr>
<tr>
<td>gallic acid</td>
<td>60</td>
<td>250</td>
<td>4</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>380</td>
<td>760</td>
<td>2</td>
</tr>
</tbody>
</table>

*Table 12:* Chemiluminescence response of a number of important classes of analytes with standard potassium permanganate ($1 \times 10^{-3}$ M, 1% w/v sodium polyphosphate, pH 2.5) and enhanced permanganate ($1 \times 10^{-3}$ M, 1% w/v sodium polyphosphate, pH 2.5, 0.3 mM manganese(II) acetate).
The manganese(II) enhanced potassium permanganate chemiluminescence detection was additionally applied post column separation. A mixture of adrenergic amines (octopamine, synephrine, tyramine, N-methyltyramine and hordenine) were separated by HPLC and detected with the new reagent (figure 31). The method for the separation of four adrenergic amines was developed earlier (see chapter 2). The addition of N-methyltyramine required minimal adjustment to the method to achieve the separation of the five adrenergic amines.

**Figure 31:** Separation and chemiluminescence detection of 5 adrenergic amines – octopamine (1), synephrine (2), tyramine (3), n-methyltyramine (4) and hordenine (5) – with the manganese(II) enhanced permanganate (A) and standard permanganate (B) reagents.
Limits of detection for octopamine, synephrine, tyramine, n-methyltyramine and hordenine were improved by over an order of magnitude (5x10^{-8} M, 4x10^{-8} M, 7x10^{-8} M, 8x10^{-8} M, 1x10^{-7} M, respectively). Flow injection methodology was utilized to obtain detection limits of 1.2x10^{-9} M for synephrine with the enhanced reagent, representing an order of magnitude improvement from previously optimized conditions. Detection limits for morphine, using flow injection analysis, were also improved to 4.6x10^{-11} M, which is an improvement to all previous limits of detection established for this reagent.

3.8 A new way of preparing the manganese enhanced permanganate reagent

The results discussed above have shown that the new permanganate/manganese(II) reagent provides significant enhancement of the chemiluminescence reaction compared to the standard acidic potassium permanganate reagent. However, the disadvantage of this new reagent is that it requires over 12 hours of standing time to equilibrate prior to analysis.

Analysis of absorption spectra for 0.1 mM KMnO4 (with 1% (w/v) sodium polyphosphate, adjusted to pH 2.5, and 0.03 mM manganese(II) sulphate), measured every 30 minutes for 48 hours, shows a decrease in absorption at 525 nm, and between 325 nm and 385 nm. This decrease is attributed to the consumption of manganese(VII) (figure 32). The depletion of permanganate is accompanied by the formation of manganese(III) complexes (seen on the
spectrum as the relatively large increase in absorbance below 300 nm, characteristic of these complexes).

Figure 32: Absorption spectra for 0.1 mM KMnO4 (with 1% (w/v) sodium polyphosphate, adjusted to pH 2.5) measured every 30 min for 48 hours following the addition of 0.03 mM manganese(II) sulphate (only every 6th spectrum shown, first spectrum is shown in bold, final spectrum is yellow).

The absorption spectrum, when obtained using the conditions employed by Perez-Benito et al. has shown that without the presence of polyphosphates, the reduction of permanganate by sodium thiosulphate favours the formation of
soluble (colloidal) manganese(IV) complexes (figure 33). This conclusion is reached by noting the significant increase in the absorption at 425 nm.

Interestingly, when 1% (w/v) sodium polyphosphate is added to potassium permanganate prior to the reduction by sodium thiosulphate (figure 34), the reaction again favours the formation of manganese(III) complexes. The shift in the isosbestic point from approximately 320 nm (figure 32) to 297 nm (figure 34) is caused by the different stoichiometric ratios of permanganate to Mn(III) in the two reactions. When permanganate is reduced by manganese(II), only 20% of the manganese(III) product is derived by the oxidant, whereas when thiosulphate is the reductant, permanganate is the only source of manganese(III).
**Figure 33:** Absorption spectra shows stepwise addition of sodium thiosulphate (18.8 mM, 1 mL per step) into KMnO$_4$ (0.5 mM, 2 L). The first spectrum is shown in bold.

Reduction of permanganate with thiosulphate, as shown by these experiments, provides a near-instantaneous approach for the preparation of the permanganate/manganese(III) reagent compared to using manganese(II), which requires a minimum of 12 hours to react with permanganate. Additional work by colleagues has demonstrated that a reagent prepared by mixing 1.9 mM potassium permanganate (containing 1% (w/v) sodium polyphosphate, pH 2.5) with 0.6 mM sodium thiosulphate is a viable alternative to manganese(II) as an enhancer.$^{144}$
Figure 34: Stepwise addition of sodium thiosulphate into KMnO₄ containing 1% (w/v) sodium polyphosphate. The first spectrum is shown in bold.
4. Conclusion

A 6-month old solution of potassium permanganate, which had been exposed to light, was shown to elicit a greater chemiluminescence signal than a freshly prepared solution of the reagent, in some cases. Further studies revealed that manganese(II), formed by the reduction of manganese(VII) over time and in the presence of light, was responsible for the observed enhancement.

Addition of manganese(II) to permanganate allowed for a greater control of reaction rates and an enhancement of chemiluminescence emission intensities. Manganese(II) catalysis has been shown to improve sensitivity and compatibility with flow analysis techniques, such as flow injection analysis and liquid chromatography. As a result, superior figures of merit for existing procedures can be obtained, as well as the ability to extend this mode of detection to new analytical applications.

Further investigations have revealed that the reduction of manganese(VII), attained by adding manganese(II) to the reagent, forms manganese(III) complexes when sodium polyphosphate is present in the potassium permanganate reagent. This reaction requires a minimum of 12 hours to complete, however using sodium thiosulphate to reduce potassium permanganate has been shown to be a viable alternative to manganese(II), and provides a near-instantaneous method for preparation of the reagent. These results have been published in the scientific literature\textsuperscript{142-144}. 
Chapter 4

Investigation into the relationship of analyte structure and chemiluminescence emission

1. Introduction

2. Experimental

3. Results and Discussion
   1. Selection of the analytes
   2. Stability study
   3. Simple aminophenols with standard permanganate
   4. Simple aminophenols with permanganate/manganese(II)
   5. Kinetic analysis of aminophenolic compounds
   6. Chemiluminescence investigations of additional structurally similar compounds

4. Conclusion
1. Introduction

Acidic potassium permanganate has been used as a chemiluminescence reagent for almost a century. The first analytical use of the reagent, however, was reported in 1972 by Nikolaevsky and Miroshnikova\textsuperscript{163}. The reagent was used to detect gas resistance in plants. Although acidic permanganate has been utilised as an analytical reagent for many applications (described in chapter 1), until recently the nature of the emitting species and the mechanism of the reaction was unknown.

Over the years several excited-state intermediates have been postulated, including manganese(II) or a complex thereof, singlet oxygen, sulphur dioxide, nitric oxide and molecular oxygen\textsuperscript{14,39-49}. In 2002, work by Barnett\textsuperscript{115} et al. provided chemical and spectroscopic evidence to show that the chemiluminescence is independent of the analyte, and originates from excited manganese(II).

The intense red emission from this reagent, upon reaction with various organic compounds, has been identified as a $^4T_1 \rightarrow ^6A_1$ phosphorescence from excited state manganese(II)*\textsuperscript{115}. More recently the mechanism for the reaction has been elucidated. Hindson\textsuperscript{164} et al. have shown the reaction involves the reduction of manganese(VII) to a polyphosphate stabilised manganese(III) species, generating a radical analyte oxidation product. The subsequent reaction between the manganese(III) and the radical forms an electronically excited manganese(II)* species, which upon relaxation to ground state emits the characteristic red light ($\lambda_{\text{max}} = 689 \pm 5$ nm) associated with permanganate chemiluminescence.
A recent review has shown organic compounds containing phenols within their structure as good candidates for the chemiluminescence reaction with the acidic potassium permanganate reagent. Additionally, there is some evidence that suggests phenolic compounds which also contain amine functional groups tend to elicit more intense luminescence. In this chapter, work is presented which examines the chemical structure of various closely related phenolic amines, and their effect on the chemiluminescence reaction with acidic potassium permanganate. The impact of the organic analytes is considered from a mechanistic point of view, and possible future analytical applications are discussed.
2. Experimental

2.1 Reagents, standards and samples.

The standard acidic potassium permanganate reagent ($1 \times 10^{-3}$ M) was prepared by dissolving potassium permanganate (Chem-Supply, Gillman, SA, Australia) in a 1% (w/v) sodium polyphosphate (+80 mesh; Sigma-Aldrich, Castle Hill, NSW, Australia) solution and adjusted to pH 2.5 with sulphuric acid. The enhanced permanganate reagent consisted of preparing a solution of standard acidic potassium permanganate (1 mM, 1% w/v sodium polyphosphate, pH 2.5) and adding to it the desired amount of manganese(II) acetate or manganese(II) sulphate, as indicated. This reagent was prepared and stored for 24 hours, to ensure the solution was at equilibrium, prior to analysis.

The analytes were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Stock solutions were prepared immediately prior to analysis and to a concentration of $1 \times 10^{-3}$ M in distilled water.

2.2 Instrumentation

Flow injection analysis manifold was constructed as described in chapter 2 of this thesis (figure 6, system 3), and contained a sample loop volume of 70 µL. The chemiluminescence signal was collected using an ‘e-corder 410’ data acquisition system (eDAQ, Denistone East, NSW, Australia).
Stopped flow experiments involved a flow injection analysis setup with a programmable dual syringe pump and an ‘e-corder 410’ data acquisition system, as described earlier (chapter 3 of this thesis).

UV-Visible absorption spectra were obtained using a Cary 300 Bio UV-visible spectrophotometer (Varian, Mulgrave, Australia) with 10 mm pathlength, sealable quartz cuvettes (Starna, Baulkham Hills BC, NSW, Australia).
3. Results and Discussion

3.1 Selection of the analytes

Experimental data indicates a disposition for phenolic compounds to be able to meet the requirements for chemiluminescence with acidic potassium permanganate. A similar character is thought to prevail with phenolic anilines\textsuperscript{165,166}. In studying the relationship between the analyte structure and its chemiluminescent response, a series of simple organic compounds, encompassing either a phenol or an aniline (or both) moieties (figure 35) has been investigated. Previously established optimized conditions for potassium permanganate chemiluminescence (1 mM KMnO\textsubscript{4}, 1% sodium polyphosphate, pH 2.5 adjusted with sulphuric acid) were employed with all analytes in question. In addition to this “standard” permanganate reagent, a recently developed acidic potassium permanganate/manganese(III) reagent (described in greater detail in chapter 3 of this thesis) was also used in this study. The above stated parameters may not be optimized for each individual analyte; however, by maintaining constant reagent conditions, differences in the chemiluminescent response can be attributed solely to individual analyte structure.
Several other compounds were selected to provide additional data that may be helpful when determining any trends emerging from the initial analytes. These supplementary compounds are shown in figure 36.
Figure 36: Structures of closely related organic compounds selected for additional chemiluminescence comparison.
3.2 Stability study

The stability of these compounds was investigated, as aminophenols are known to polymerise\(^{167}\). A visible colour change was observed within 12 to 24 hours of preparation for some of the analytes. This observation was made when the analytes were prepared in both an aqueous and a methanol solution. For example, while no visible change was found to have occurred for 1,3-phenylenediamine after 7 days, 1,2-phenylenediamine, on the other hand, was noted to have changed from a transparent to a yellow solution in the same time frame.

The initially clear aqueous solution of 4,6-diaminoresorcinol changes to a light pink colour in approximately one hour. This physical change was investigated with time of flight mass spectroscopy (TOF-MS). The exact mass of this compound is 140.06 amu. The mass spectrum, 1 hour after preparation of the analyte, shows a peak at 139.049 amu in positive mode, and 137.036 amu in negative mode, indicating the parent molecule is 138 amu - two mass units less than the molecular weight of 4,6-diaminoresorcinol. It is speculated that this compound starts oxidising in solution, possibly in the presence of light. A possible oxidation product is shown in figure 37. This product has an exact mass of 138.042 amu and fits the observed mass spectrum data.
Figure 37: Proposed oxidation of 4,6-diaminoresorcinol in an aqueous solution.

If 4,6-diaminoresorcinol is polymerising, the product is expected to be either a condensation polymer or a substitution type polymer. The mathematical description for the mass of a condensation polymer is shown in equation 1 below, and a substitution type polymer is described in equation 2.

**Equation 1:** Expected mass = \((n \times MW) - 18(n - 1)\)

**Equation 2:** Expected mass = \((n \times MW) - 2(n - 1)\)

Therefore, if the oxidation product of 4,6-diaminoresorcinol dimerises, it is anticipated that the mass of this dimer will be 258.08 amu (if the dimer is a product of a condensation polymer) or 274.08 amu (if the dimer is a substitution type polymer). The mass spectrum shows a peak at 275.075 amu in the positive
mode. A concordant peak was also observed in the negative mode (273.066 amu), suggestive of the formation of a substitution type dimer. A peak indicating the formation of a trimer (expected at 410.130 amu) was not observed.

It should be mentioned that a freshly prepared solution of 4,6-diaminoresorcinol shows a parent peak at 141.08 amu in the positive mode (expected peak for 4,6-diaminoresorcinol), which decreases over time. The decrease in this peak is coupled with the increase in the peak at 139.05 amu, showing the oxidation of the analyte over time (figure 37). After 24 hours almost all of the analyte has oxidised and/or dimerised.

Some other aminophenols were also observed to undergo colour change over time, and were investigated with TOF-MS (table 13). Overall, it was noted that while some compounds change in solution over time, they were sufficiently stable at least over the first hour, which was deemed suitable for the purpose of this study. An exception is 4,6-diaminoresorcinol, which was found to rapidly auto-oxidise. Consequently, conclusions about the effect on the chemiluminescence from this compound need to take into consideration its limited stability in aqueous solution. As a countermeasure, this compound – and all others involved in this study – was always prepared immediately prior to detection, such that the time between preparation and analysis was minimal.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-aminophenol</td>
<td>Shows no visually observable physical change over 3 hours of monitoring. Clear solution with a light yellow tinge darkens over 24 hours. TOF-MS data shows no change over the first hour.</td>
</tr>
<tr>
<td>3-aminophenol</td>
<td>No physical change observed up to 7 days. Chemiluminescence calibration after 24 hours shows no difference in intensity compared to the freshly prepared solution. Appears stable in solution.</td>
</tr>
<tr>
<td>4-aminophenol</td>
<td>No apparent change in the first hour. Initially a clear solution, after 24 hours a dark brown discoloration is noted with formation of precipitate. TOF-MS data shows no apparent change over the first hour. First evidence of polymerisation is observed with a 5 hour old solution.</td>
</tr>
<tr>
<td>2,3-diaminophenol</td>
<td>Solution remains clear up to 4 days. Some colour augmentation is observed about 7 days after preparation.</td>
</tr>
<tr>
<td>2,4-diaminophenol</td>
<td>After one hour solution shows faint signs of colour darkening. Visually the solution changes from pink to a dark-red colour after 24 hours. Mass spectrum data shows no change in the first hour. First evidence of dimerisation is observed in the spectrum from a 5 hour old solution.</td>
</tr>
<tr>
<td>4-aminoresorcinol</td>
<td>No apparent physical change after 24 hours. Compound appears stable in solution for the purpose of this study.</td>
</tr>
<tr>
<td>4,6-diaminoresorcinol</td>
<td>Very slight colour change observed in the first hour. Initially the solution is clear, within 2 hours a faint light-pink colour is noted, which dims to red in 24 hours, and further darkens to brown after 4 days. Mass spectrum data indicates that after one hour the compound is mostly unoxidised, but some portion of the analyte is oxidised. The compound is almost completely oxidised within 24 hours.</td>
</tr>
</tbody>
</table>

Table 13: Summary of the investigation into the stability of aminophenols in aqueous solution. Those samples for which a colour change was observed were further analysed with Time-of-flight mass spectrometry (TOF-MS).
3.3 Simple aminophenols with standard potassium permanganate reagent

The chemiluminescence response of each of the above organic compounds was compared. Each analyte was diluted to a concentration of $1 \times 10^{-5}$ M, and the reagent conditions were unchanged (1mM KMnO$_4$, 1% w/v sodium polyphosphate, pH 2.5 adjusted with sulphuric acid). First the ortho-substituted compounds were evaluated with the standard permanganate reagent, as illustrated by figure 38.

![Chemiluminescence Graph]

**Figure 38**: A comparison of the chemiluminescence intensity obtained from the ortho-substituted compounds and the standard potassium permanganate reagent.
The graph shown in figure 38 indicates that amine groups on the benzene ring provide a better chemiluminescence response than hydroxyl groups. In fact, a 3-fold increase in signal intensity is observed when a hydroxyl group of pyrocatechol is replaced by an amine substituent. Replacing both hydroxyl groups with amines further enhances the observed emission.

A comparison of the meta-substituted analytes is shown in figure 39. The trend observed with these analytes is alike that noted with the ortho-substituted compounds. When a hydroxyl group of resorcinol is substituted with an amine, a 13-fold increase in the chemiluminescence response was observed. A further 2.5-fold enhancement in the signal intensity is seen when both substituents are amines (1,3-phenylenediamine). Interestingly, the emission intensity with these compounds is significantly lower than with the ortho-substituted compounds (see figure 40).
Figure 39: Emission intensity of meta-substituted compounds with standard permanganate.

Figure 40: Chemiluminescent emission intensities for the α- and m- substituted compounds
A five to ten fold lower chemiluminescence intensity was observed with the *meta* arranged isomers (figure 40), showing the importance of analyte structure. Hindson *et al.* have shown that the mechanism for this reaction involves the production of a radical intermediate, derived from the analyte as manganese(VII) is reduced to manganese(III). A subsequent reaction between the radical and manganese(III) yields an excited state manganese(II)* species, which then emits a photon, returning to ground state. Hydroxyl and amino groups are both *ortho-* and *para-* directing activators of the benzene ring. Moreover, the radical intermediate can be resonance stabilised, by moving the odd electron through the *ortho* and *para* positions on the benzene ring (as illustrated in figure 41). Therefore the higher signal intensity observed with *ortho*-substituted compounds is likely due to the production of a more resonance stabilised analyte radical, which would not be the case with the *meta* configurations of the isomers. The data also indicates that amine moieties have a more positive influence on the chemiluminescence intensity than the hydroxyl counterparts.

![Figure 41](image)

*Figure 41:* Resonance stabilisation of a radical intermediate is achieved by the movement of the electron through the *ortho* and *para* positions on the ring.
The chemiluminescence response for the *para*-substituted isomers of the above compounds was also obtained. The data, shown in figure 42, supports the emerging trend observed thus far, with the *para*-dihydroxy benzene (hydroquinone) yielding the lowest response, and the diaminebenzene producing the largest response. Indeed, 1,4-phenylenediamine gave the largest response by a factor of four. Additionally, the detected chemiluminescence for the *para* configured compounds is also significantly greater than the *meta* counterparts (10 to 30 fold higher emission intensity).

![Chemiluminescence graph](image)

**Figure 42:** Emission intensity of *para*-substituted compounds with standard permanganate.
Interestingly, it appears that anilines yield a more intense chemiluminescence signal than phenols, which is somewhat surprising as this reagent has primarily been used for the detection of phenolic compounds. Amine radicals are more stable than hydroxide radicals, thus the rate of production of these radicals would be higher, and therefore more suited to flow analysis detection, where reaction kinetics play a significant role in the resulting chemiluminescence intensity.

### 3.4 Simple anilines with potassium permanganate/manganese(II) reagent

In addition to the “standard” potassium permanganate, chemiluminescence of the nine compounds (figure 35) was also measured with the recently developed manganese(VII)/manganese(II) reagent (discussed in greater detail in chapter 3). A comparison of the ortho-substituted compounds with this reagent is shown in figure 43. Unlike the observations made using “standard” potassium permanganate, the maximum signal intensity in this case is observed with 2-aminophenol. The intensity observed for pyrocatechol is 3-times greater with the permanganate/manganese(II) reagent compared to the standard reagent. A 20% increase in intensity is noted for 2-aminophenol, however with 1,2-phenylenediamine a 50% decrease was found (see figure 43).
Figure 43: Chemiluminescence comparison of the standard potassium permanganate and enhanced permanganate/manganese (II) reagent with pyrocatechol, 2-aminophenol and 1,2-phenylenediamine.

The results obtained for meta compounds mimicked those obtained with the standard permanganate reagent, whereby the response improved as the number of amine substituents increased (see figure 44). Interestingly however, the signal was drastically more intense than that observed with the standard reagent, as depicted in table 14.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Signal with standard permanganate (mV)</th>
<th>Signal with permanganate / manganese(II) (mV)</th>
<th>Enhancement factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resorcinol</td>
<td>3</td>
<td>273</td>
<td>71</td>
</tr>
<tr>
<td>3-aminophenol</td>
<td>50</td>
<td>1513</td>
<td>30</td>
</tr>
<tr>
<td>1,3-phenylenediamine</td>
<td>131</td>
<td>6664</td>
<td>51</td>
</tr>
</tbody>
</table>

**Table 14:** Chemiluminescence emission enhancement for *meta*-substituted compounds

The three *para*-substituted compounds, hydroquinone, 4-aminophenol and 1,4-phenylenediamine, depicted in figure 42, show an approximate 2-fold increase in signal intensity for hydroquinone and 4-aminophenol, but for 1,4-phenylenediamine a decrease of a similar magnitude was observed, akin to the observations made with *ortho*-substituted compounds with this reagent.
Figure 44: Chemiluminescence intensities for nine structurally similar compounds obtained using a standard and enhanced permanganate reagent.

With the “standard” permanganate reagent the ortho- and para- analytes were found to yield significantly more light than the meta isomers. This trend was not followed when the reagent was enhanced with manganese(III). For example, with “standard” permanganate the chemiluminescence of the ortho-aminophenol is approximately 5-fold more intense than the meta-aminophenol, but when manganese(III) catalysed reagent is used, the meta-aminophenol signal is enhanced by a factor of 30, thus with this reagent it is the meta-
substituent which produces a greater chemiluminescence output by a factor of around 6.

In chapter 3 of this thesis it was shown that when manganese(III) is generated from potassium permanganate, achieved by the addition of either a manganese(II) salt or sodium thiosulphate, the otherwise slow oxidation of adrenergic amines is significantly enhanced. The increase in the reaction rate of these compounds was accompanied by large enhancements in signal intensity, similar to those noted in table 14. It is therefore speculated that the rate of oxidation of resorcinol, 3-aminophenol and 1,3-phenylenediamine is very slow normally, however with the permanganate/manganese(III) reagent, there is an increase in the reaction kinetics, resulting in the observed emission intensities.

Despite the similarity of the nine compounds investigated, there were vast differences in the chemiluminescence response of these analytes (table 15). Discrepancy in the signal intensity of isomers with differing configurations was found to vary by up to 20-fold. For example, pyrocatechol (ortho-substituted dihydroxybenzene) was noted to be 20 times more intense then the meta isomer, but its emission was approximately 2-fold weaker than the para isomer, when using standard potassium permanganate to initiate the reaction. Similarly the chemiluminescence signal of compounds where one or both substituents were anilines was also observed to be dependent on the structural arrangement of the moieties. The data presented in table 15 suggests that analyte structure plays a significant role in chemiluminescence emission. The signal intensity of any one particular analyte is influenced not only by the presence of hydroxyl
and/or aniline groups, but also by the relative structural arrangement of these groups.

<table>
<thead>
<tr>
<th>Substituents</th>
<th>Configuration</th>
<th>“Standard” KMnO₄</th>
<th>Thiosulphate enhanced</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OH – OH</strong></td>
<td><em>Ortho</em></td>
<td>100*</td>
<td>285</td>
</tr>
<tr>
<td></td>
<td><em>Meta</em></td>
<td>5</td>
<td>363</td>
</tr>
<tr>
<td></td>
<td><em>Para</em></td>
<td>231</td>
<td>505</td>
</tr>
<tr>
<td><strong>OH – NH₂</strong></td>
<td><em>Ortho</em></td>
<td>361</td>
<td>483</td>
</tr>
<tr>
<td></td>
<td><em>Meta</em></td>
<td>67</td>
<td>2008</td>
</tr>
<tr>
<td></td>
<td><em>Para</em></td>
<td>387</td>
<td>971</td>
</tr>
<tr>
<td><strong>NH₂ – NH₂</strong></td>
<td><em>Ortho</em></td>
<td>537</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td><em>Meta</em></td>
<td>174</td>
<td>8839</td>
</tr>
<tr>
<td></td>
<td><em>Para</em></td>
<td>2019</td>
<td>1182</td>
</tr>
</tbody>
</table>

**Table 15:** Relative signal intensities with pyrocatechol (*ortho*-dihydroxybenzene) set to 100%.

### 3.5 Kinetic analysis of related aminophenolic compounds

The kinetic profile of all of the compounds investigated thus far was obtained by means of stopped-flow methodology. The reagent and analyte were merged and propelled into the reaction chamber, where they were held so that the entire chemiluminescence emission as a function of time could be captured. The chemiluminescence intensity over time profiles of pyrocatechol and hydroquinone, containing *ortho* and *para* substituted hydroxyl groups,
respectively, are shown in figure 45. These analytes were merged with standard potassium permanganate reagent.

![Graph showing the oxidation of pyrocatechol and hydroquinone](image)

**Figure 45:** Oxidation of pyrocatechol and hydroquinone with standard potassium permanganate (1 mM, 1% (w/v) sodium polyphosphate, pH 2.5). All analytes are 1x10^{-5} M.

A reaction between acidic potassium permanganate and hydroquinone was noted to be more intense than that between the reagent and pyrocatechol, using flow injection analysis. From figure 45, it is evident that the oxidation of hydroquinone occurs at a slightly faster rate, as noted by the sharp rise of the hydroquinone peak compared to the pyrocatechol peak. As mentioned earlier,
reaction rate has a considerable influence on emission intensity with flow analysis based systems. As the system is flow based, a sample is continuously propelled through the reaction chamber and spends a limited amount of time in front of the detector. The overall emission intensity for a sample is the emission intensity that is detected in the flow cell, therefore if a reaction is too slow, a large portion of the emission will occur outside the reaction chamber and will not be observed by the detector.

The oxidation of the meta-substituted resorcinol with the standard permanganate reagent is significantly slower than both pyrocatechol and hydroquinone (figure 46). The intensity of resorcinol is, on average, 40-fold weaker than the signal emission from hydroquinone, based on peak height and peak area, using flow injection analysis. Resorcinol is a very slow reductant of potassium permanganate. The maximum emission is observed from 10 seconds through to 40 seconds, much later than the emission maxima for hydroquinone and pyrocatechol (1.8 sec and 2.1 sec, respectively). Using FIA, as resorcinol approaches its maximum emission with acidic potassium permanganate; it has passed the detector, and is being propelled to waste. Accordingly, only a small fraction of light is actually being detected in the flow cell by the photomultiplier tube.

When the manganese(III) catalysed potassium permanganate reagent is used, a emission intensity enhancement of approximately 2.5-fold is noted for pyrocatechol and hydroquinone, however, for resorcinol the chemiluminescence signal is 40-70 times greater. The amount of enhancement observed was found
to depend on analyte concentration, instrumental setup and the preparation of the reagent (manganese(III) can be generated in the permanganate reagent either by addition of a manganese(II) salt, or by reduction of permanganate with thiosulphate), as illustrated by figure 47 and in table 16.

![Graph showing chemiluminescence intensity-time profile](image)

**Figure 46**: Chemiluminescence intensity-time profile of resorcinol with “standard” potassium permanganate (y-axis on the left) and manganese enhanced potassium permanganate (y-axis on the right).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Resorcinol</th>
<th>3-aminophenol</th>
<th>1,3-diaminophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>Enhancement factor</td>
<td>Enhancement factor</td>
<td>Enhancement factor</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$ M</td>
<td>n.a</td>
<td>21.2</td>
<td>n.a</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$ M</td>
<td>n.a</td>
<td>14.3</td>
<td>58.2</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$ M</td>
<td>45.0</td>
<td>16.7</td>
<td>27.4</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$ M</td>
<td>63.8</td>
<td>14.7</td>
<td>101.4</td>
</tr>
</tbody>
</table>
Table 16: Chemiluminescence signal enhancement observed when “standard” permanganate is switched to manganese(III) catalysed permanganate (manganese(III) was generated by the addition of manganese(II) acetate), and its dependence on analyte concentration.

![Chemiluminescence signal enhancement chart]

**Figure 47:** The method by which manganese(III) catalysed potassium permanganate reagent is generated has an influence on the chemiluminescence signal enhancement observed.

The significant enhancement of the chemiluminescence signal for resorcinol with the manganese enhanced permanganate can be primarily attributed to the vast improvement in the rate of this reaction, as illustrated in figure 46. With standard permanganate it takes approximately 10 s. for the reaction to reach its
maximum emission intensity. The intensity stays at the maximum level for approximately 35 s. before it starts decreasing. The total time required for the reaction to return to baseline is 120 s. With manganese enhanced permanganate this reaction takes about 3 seconds to reach the maximum intensity, and by the 10 second mark the reaction has nearly concluded – representing a momentous difference in the rate of the reaction of resorcinol with the two reagents.

![Graph](image)

**Figure 48:** The chemiluminescence-time profiles of pyrocatechol and hydroquinone with standard and enhanced potassium permanganate reagent.

The rate of oxidation of hydroquinone and pyrocatechol is also enhanced by the permanganate/manganese reagent (figure 48). In terms of the signal
enhancement, the emission of hydroquinone is 2 times more intense, while pyrocatechol is 2.5 times more intense. These increases pale in comparison to the near two-orders of magnitude enhancement observed with resorcinol. The very slow oxidation of resorcinol becomes much quicker, but with hydroquinone or pyrocatechol, the initial reaction is already relatively quick, and only a minor increase in the rate of the reaction for the latter two compounds occurs with the enhanced reagent. In fact, the rate of oxidation of hydroquinone with the standard permanganate reagent is faster than the oxidation of resorcinol with the enhanced reagent (figure 49).

![Graph showing the rate of oxidation comparison between resorcinol and hydroquinone](image)

**Figure 49:** The rate of oxidation of hydroquinone with "standard" permanganate compared to the reaction rate of resorcinol with the manganese(III) catalysed permanganate reagent.
A comparison of \textit{ortho}-substituted compounds; pyrocatechol, 2-aminophenol and 1,2-phenylenediamine (figure 50), shows an increase in reaction kinetics with increasing number of aniline over hydroxyl groups with the standard reagent. That is, 1,2-phenylenediamine reached the emission maximum faster than 2-aminophenol, which in turn was quicker than pyrocatechol. The same comparison with the manganese enhanced reagent shows these compounds further increase their overall kinetic rate, with the exception of 1,2-phenylenediamine, which maintains the same rate. A similar trend was observed with the \textit{para}-substituted compounds. The increase in the speed of these reactions translates to an approximate two-fold increase in the observed chemiluminescence when the compounds are detected with flow injection analysis.
Figure 50: Comparison of reaction kinetics for ortho-substituted compounds with “standard” permanganate and manganese(III) catalysed potassium permanganate.

During the course of the chemical reaction between an analyte and acidic potassium permanganate, an intermediate radical product is stabilised through the ortho and para positions on the benzene ring, which is not the case with meta compounds (see figure 41), consequently the rate at which these analytes are oxidised is greatly reduced (figure 51). Even so, the oxidation of 3-aminophenol and 1,3-phenylenediamine is much faster, relatively, than resorcinol. Still, the time required to reach 90% of the peak area, and therefore 90% of the entire chemiluminescence emission is between 30-40 seconds for 3-
aminophenol and 1,3-phenylenediamine. Substantially increasing the reaction kinetics of these compounds by using manganese(III) catalysed potassium permanganate (figure 52) has resulted in a major increase in the signal intensity. With the “standard” reagent (figure 51) at the 10 second mark 3-aminophenol and 1,3-phenylenediamine have just passed the point of maximum emission, and resorcinol has just reached that point. When manganese(III) catalyses the reaction (figure 52) at that same 10 second mark all three reactions have concluded. With regards to the two amine compounds, the reaction is nearly complete after just 3 seconds.

Figure 51: Kinetic profile for the reaction of meta-substituted compounds with “standard” potassium permanganate reagent.
Figure 52: Kinetic profile for the reaction of meta-substituted compounds with the enhanced potassium permanganate reagent.

3.6 Chemiluminescence investigations of additional structurally similar compounds

Several additional compounds (figure 36) were also determined with “standard” and enhanced potassium permanganate. A comparison of 2-aminobenzyl alcohol and 3-aminobenzyl alcohol with the closely related 2-aminophenol and 3-aminophenol is summarised in table 17. Thus far the results have shown aniline compounds in general produce a greater chemiluminescence signal than phenols due to their faster kinetics. Interestingly, comparing 2-aminophenol and 2-aminobenzyl alcohol, there is a two order of magnitude difference in emission between the two compounds, based on flow injection analysis with standard
permanganate. The two compounds differ in that the hydroxyl group of 2-aminobenzyl alcohol is not directly attached to the benzene ring but is one carbon atom away from the ring. A similar result is obtained when the meta compounds (3-aminophenol and 3-aminobenzyl alcohol) are compared, with a 20-fold decrease in chemiluminescence signal observed with the compound which does not have a hydroxyl group directly bound to the benzene ring. The enhanced permanganate reagent does increase the observed emission by about 5-8 fold for the two alcohols, however even with this enhancement the detected emission is still more than an order of magnitude weaker than the emission of 2-aminophenol with the standard reagent.

<table>
<thead>
<tr>
<th>Compound</th>
<th>“Standard” KMnO₄</th>
<th>Enhanced KMnO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-aminophenol</td>
<td>100*</td>
<td>93.9</td>
</tr>
<tr>
<td>3-aminophenol</td>
<td>9.0</td>
<td>150.3</td>
</tr>
<tr>
<td>2-aminobenzyl alcohol</td>
<td>1.1</td>
<td>6.9</td>
</tr>
<tr>
<td>3-aminobenzyl alcohol</td>
<td>0.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Table 17: Relative chemiluminescence signal response of several compounds with “standard” permanganate and manganese(III) catalysed permanganate. The response of 2-aminophenol with “standard” permanganate is set to 100%. 
The chemiluminescence response of other compounds is shown in figure 53. It was observed that none of the five additional compounds gave a noticeably larger chemiluminescence response with the enhanced reagent. Based on the information discussed earlier in this chapter, this would indicate that the rate of reaction of these compounds with standard potassium permanganate is already fast, thus the enhanced reagent would have minimal effect on the kinetics of said reactions and a minimal overall chemiluminescence enhancement is observed.

![Figure 53: Chemiluminescence response of five compounds with both standard and enhanced potassium permanganate.](image-url)
**Figure 54:** Chemiluminescence-time profile for several compounds with the standard potassium permanganate reagent.

As anticipated, the reaction kinetics of these compounds (figure 54) were found to be relatively quick in all cases, with the exception of 4,6-diaminoresorconol. While the overall peak profile of 4,6-diaminoresorconol required approximately 60 seconds to return to baseline, the time required to reach maximum emission was between 1 and 2 seconds, thus making the reaction relatively quick when measuring peak height using flow injection analysis. A summary of the kinetic profiles (table 18) shows that the time required to reach emission maximum had decreased for all compounds, however this did not result in any significant enhancement of the chemiluminescence signal of these compounds.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Time to reach maximum signal [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard permanganate</td>
</tr>
<tr>
<td>2,3-diaminophenol</td>
<td>0.575</td>
</tr>
<tr>
<td>2,4-diaminophenol</td>
<td>1.650</td>
</tr>
<tr>
<td>4-aminoresorcinol</td>
<td>1.250</td>
</tr>
<tr>
<td>4,6-diaminoresorcinol</td>
<td>1.825</td>
</tr>
<tr>
<td>5-amino-2-methoxyphenol</td>
<td>1.725</td>
</tr>
</tbody>
</table>

Table 18: Time required to reach maximum emission of several compounds with the standard and enhanced potassium permanganate reagents.

From the above data several interesting points were noted. The chemiluminescence response, with FIA, of 4-aminoresorcinol was approximately 8-fold higher than 4,6-diaminoresorcinol. Results from the initial nine compounds showed that substituting hydroxyl groups with anilines improved the chemiluminescence response, however, in this case the addition of a supplementary amine substituent resulted in a significant decrease in the chemiluminescence signal. The crowded arrangement, with four moieties attached to the benzene ring, is thought to be the cause of the reduced chemiluminescence signal.

Furthermore, the emission intensity of 2,3-diaminophenol was about 1 order of magnitude less than the intensity recorded for 2,4-diaminophenol. Relative to
each other, the two anilines are ortho substituted in the case of 2,3-diaminophenol, while the 2,4-diaminophenol is meta substituted (figure 36). The signal attained for 2,4-diaminophenol was approximately double the signal for 4-aminoresorcinol. These two compounds are similar, with the former having an amine group in the 2\textsuperscript{nd} and 4\textsuperscript{th} position relative to the hydroxyl group, while the later compound has two hydroxyl groups in the 2\textsuperscript{nd} and 4\textsuperscript{th} position relative to the amine moiety. This further indicates that amine groups do yield a more intense chemiluminescence emission than the hydroxyl counterparts.

The arrangement of anilines which produced the highest signal was in the 2\textsuperscript{nd} and 4\textsuperscript{th} position relative to the third substituent on the benzene ring, which was somewhat surprising, as this configuration in a benzene molecule which only has the two aniline substituents produced the poorest result. Finally, a benzene molecule which has four substituents was also found to yield poor chemiluminescence with potassium permanganate. These results (figure 55) indicate that there is a strong relationship between analyte structure and the resultant chemiluminescence signal. It was observed that type of substituent, the number of substituents and the arrangement of substituents around the benzene ring all play a significant role in the chemiluminescence emission intensity of an analyte.
Figure 55: Chemiluminescence comparison. 3-aminophenol is a di-substituted benzene, 2,4-diaminophenol and 4-aminoresorcinol are tri-substituted benzenes and 4,6-diaminoresorcinol has 4 substituents on the benzene ring. All compounds are $1 \times 10^{-5}$ M.
4. Conclusion

Investigations into the relationship between analyte structure and the chemiluminescence intensity with the acidic potassium permanganate reagent have revealed a preference for ortho- and para- substituted phenols, aminophenols and phenylenediamines over the meta- substituted isomers.

Further studies noted the meta- substituents were oxidised at a significantly slower rate by potassium permanganate. A significant enhancement of these compounds can be achieved by increasing the concentration of manganese(III) in the potassium permanganate reagent, by reducing the permanganate either with manganese(II) salts or sodium thiosulphate.

These findings highlight the importance of instrumental configuration, including solution flow, efficient mixing and reaction kinetics of each analyte, to the observed chemiluminescence intensity. The results also demonstrate the potential to fine-tune the selectivity of potassium permanganate reactions toward a certain type of compound, or even a specific positional isomer. The results presented in this chapter have been published in the scientific literature\textsuperscript{168}. 
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