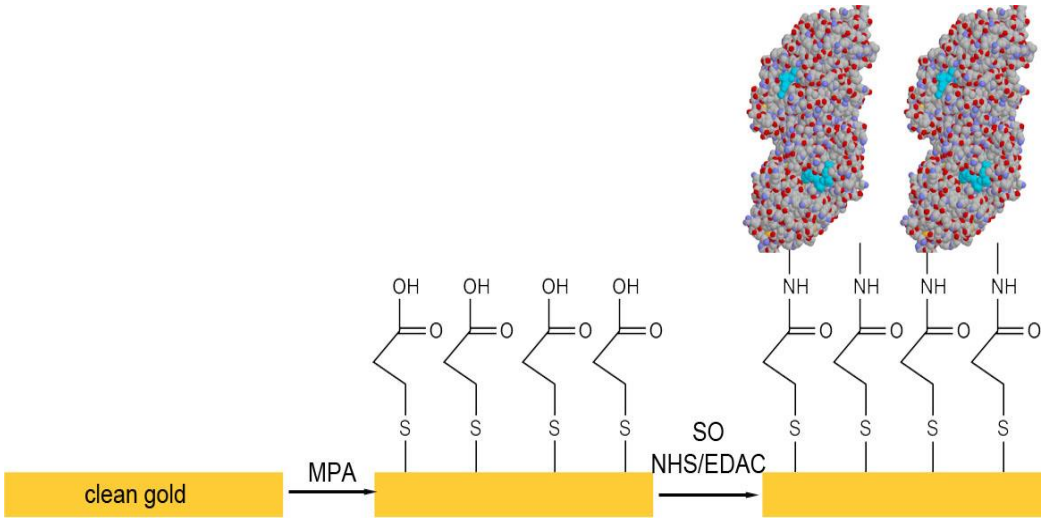
# Rapid Measurement of Sulphur Dioxide in the Winery




**FINAL REPORT to**
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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Principal Investigator: **Professor Joe Shapter**

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# Rapid Measurement of Sulphur Dioxide in the Winery

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**Abstract:**

In this work, an amperometric biosensor has been designed for the fast detection of sulphite in aqueous solutions. A sulphite oxidation peak at is observed using cyclic voltammetry and exhibits a linear peak increase with sulphite concentration. A calibration curve (current versus sulphite concentration) was produced to characterise the biosensors. However, the complex wine solutions revealed no observable signal indicating that the polyphenols and/or proteins present in wine inhibited the electron transfer. With the use of polymer films, control of pH, gold nanoparticles and/or enzymes and proteins, it was hoped that the design may be improved increasing the stability and signal. However, work to date has not been successful in getting the sensor to work in wine or other beverages.

## 2. Executive summary:

Sulphur dioxide is added to wine at nearly every stage of production and serves as both an anti-microbial agent and an antioxidant. Currently, sulphur dioxide measurement is time consuming and costly (particularly in terms of staff time or outsourcing) and cheaper, rapid methods have long been sought. The molecular form of sulphur dioxide is heavily influenced by the pH of the solution and it exists in various forms ranging from molecular $SO_2$ at low pH to sulphite ion at high pH. As wine is kept at a pH of 3-4, sulphur dioxide is converted to mainly bisulphite ions with a small amount of molecular $SO_2$.

Early work at Flinders demonstrated the successful detection of sulphite in standard solutions using an enzymatic electrochemical approach. This project built on that work to develop a robust, simple, reproducible construction protocol for the sensor and then test all analytical properties of the sensor both using standard solutions and representative wine samples. The successful development of the sensor could lead to a dramatic reduction in the effort required at wineries to measure $SO_2$ concentrations.

The construction of the biosensor with surface attachment chemistry on gold was done successfully and proved to be very reproducible. In short a small molecule with an affinity for the substrate was first attached to the surface. These molecules provided attachment points for the active species in the sensor and these biologically active compounds were attached using standard coupling chemistry. The sensors were then tested for responses in standard aqueous solutions to determine important sensor parameters such as sensitivity, response times, reproducibility, lifetime and linearity of response.

The sensor was shown to readily detect sulphite in standard solutions down to concentrations of a few ppm. Linear responses were observed over a concentration range of at least 100 ppm. Higher concentrations to test for the upper limits of detection were not measured as this was not thought to be relevant in the wine industry. Sensors could be made reasonably routinely and were shown to be long lived. Methods to restore activity were also developed.

The use of the sensor in wine samples was unsuccessful with no signals observed. This was thought to be due to the fouling from something in the complex wine matrix. Several sources of interferents were explored including ethanol and polyphenols. The sensors still did not work without these species present so further work is required to explore the nature of the deactivation of the sensor.

Once these inferences are understood there is ready scope to build a new sensor that will work in the wine matrix. Some possible avenues forward include the use of a different enzyme or detection system, addition of an antifouling layer, controlling of the morphology of the sensor to reduce certain adsorption, building an architecture where the environment of the sensing element can be controlled and maintained more readily etc.

This work has great potential and presents the exciting possibility of developing a cheap, easy to use and reusable sensor for sulphite in wine and in fact other beverages. The successful development of such a device would have a global impact on the wine industry.

## 3. Background:

### Winery Impact Overview

Sulphite is a major issue in the wine industry for which there are no quick or convenient testing methods.[1-3] Winemakers and laboratory personnel are the major audience for adoption. The addition of sulphur dioxide during the process is critical in terms of dose and timing, and both bound and free sulphur dioxide need to be measured to ensure high wine quality. Furthermore, some consumers are allergic to sulphites which poses issues for wine producers and marketers. A rapid, high accuracy, simple method for measurement will reduce the investment required to get this critical compositional parameter in a timely manner.

The need for faster, cheaper methods to measure sulphur dioxide have long been evident, but practical solutions have been difficult to develop. Measuring sulphite/sulphur dioxide concentrations in wine is critically important to ensure high quality wines. Mismanagement of sulphite/sulphur dioxide concentrations can mean significant losses of product and hence income. This project set out to develop cheap, reliable point of production testing of sulphite to ensure high quality product is produced using the most appropriate amount of $SO_2$. Wineries would be able to do the testing on site during production eliminating the need for sending samples to dedicated off-site chemical analysis services saving both time and money.

The important end product is a test that would enable winemakers to monitor sulphite levels throughout the production process ensuring concentrations are sufficient to guarantee the preservatives beneficial characteristics while minimising the negative ones. Current testing methods include sulphite test strips, which tend to be unreliable, or titration methods which are equipment and time-intensive.

Some forms of sulphur are unpleasant in the environment potentially producing foul odours. It reacts easily with other substances to form harmful compounds, such as sulphuric or sulphurous acid and sulphate particles. Hence, minimal use of $SO_2$ and sulphur containing compounds is beneficial to the environment. The development of an on-site, reliable sensors would minimise reagent use and associated waste handling and disposal issues while ensuring more efficient use of the potential pollutant sodium metabisulphite and $SO_2$. Furthermore, reduced electricity use for analysis and fuel for transport of samples to outsourced labs will be environmentally beneficial.

Less staff time will be needed for analyses while there will be reduced OH&S risk to staff through removing the use of naked flame, glassware and hazardous substances used in the aspiration method. Reduction of $SO_2$ exposure both in production and analysis is important as sulphur dioxide can affect human health when it is breathed in. It irritates the nose, throat, and airways to cause coughing, wheezing, shortness of breath, or a tight feeling around the chest.

Most equipment to perform $SO_2$ analysis is either time-consuming (aspiration) or expensive (flow injection analysis, outsourced to an analytical laboratory). Spectroscopy can predict $SO_2$ concentration within one matrix (e.g. one wine treated several different ways), but as yet a generalised calibration capable of wide-scale prediction has not been able to be developed. Immobilisation of enzymes with specificity for particular analytes has been proven both scientifically and commercially successful (for e.g. blood glucose biosensors).

As highlighted in the GWRDC 5 Year Research and Development Plan ensuring that "…Australian wine continues to meet the rigorous consumer and market standards" is vital and can be done through the development of technical solutions to eliminate wine faults and maintain product integrity. This project undertook the basic research required to develop cheap, reliable monitoring of sulphite/sulphur dioxide concentrations at every stage of the winemaking process. This would ensure the highest quality product and minimise spoilage both during production and in the bottle.

This project also sought to "To support the development of novel and improved winemaking processes". While this project does not develop new yeasts or use microbiology, it would provide winemakers with a new tool in the winemaking process. The ease of use of the sensor might allow more frequent monitoring of sulphite levels providing winemakers with more information leading to an ability to make well informed decisions in the winemaking process. This will likely lead to the use of lower levels of $SO_2$ providing both improved consumer acceptance of the product as well as "A reduction in average winemaking cost …"

**Research Background**

Sulphite detection using enzymes has been a subject of investigation for over twenty years. Table 1 summarises various approaches that have been used. Two main enzymes are used in this work, namely sulphite dehydrogenase or sulphite oxidase. In both cases, a biological cofactor, cyctochrome c, is required. The use of the cofactor raises issues of keeping biological species in solution stable over long periods. The immobilised enzyme(s) can be encased in various systems to keep the system stable but complicates sensor construction and slows response.

The two most important issues with the enzyme biosensors lie in application in the wine industry. The first is that while sulphite detection has been demonstrated in "standard samples", it has not been detected in wine. Given the complexity of wine, this will likely be a considerable hurdle requiring the investigation undertaken in this project. The second issue is

**Table 1: Review on current biosensors developed for sulphite detecting using sulphite oxidase.**

| Enzyme | Protein | Method | Analysis | Ref |
|---|---|---|---|---|
| SUOX chicken liver 2.5 mg/mL | | Pt. electrode with PAA (0.1M) and SUOX electrodeposited | Amperometry 0.0V | 4 |
| His tagged human SUOX 48.5 mg/mL | Cyt c equine heart Sigma Aldrich | Carbon screen printed electrode modified with PEGDGE and PEI-SUOX on top | CV -0.5 to 0.1V Amperometry -0.1V | 5 |
| SUOX chicken liver 3-11 Units/mL | | Pt. disc electrode-SUOX (5units/mL) entrapment in polypyrrole (0.1M) electrodeposited | Amperometry -0.7V | 6 |
| SUOX (sigma) 100 µL | cyt c horse heart 0.006g | Microchip-SUOX and cyt c were electrostatically deposited together | Amperometry 0.3V | 7 |
| SUOX chicken liver 14 units/mL | | 0.1M tyramine with SUOX electrodeposited-5 scans | CV 0.3 to 0.9V | 8 |
| Human SUOX (10 µM) | Cyt c (20 µM) | Multilayers from solutions of pure SUOX and Cyt c | CV -0.2 to 0.2V | 9 |
| SUOX extracted from *Syzgium cumini* leaves 100 µL of 36386 units/mg | | Purified SUOX was immobilised covalently onto the AuNPs/CHIT/cMWCNTs/PANI modified Au electrode | CV 0 to 0.8V | 10 |

| | | | | |
|---|---|---|---|---|
| Wild-type SDH 21 uM (246 units/mg) | Cyt c (100 µM) | SAM of 11-mercaptoundecanol cast onto an Au electrode and subsequent SUOX and cyt c adsorption | CV -0.2 to 0.4V | 11 |
| SUOX 0.5 units | | Glass carbon electrode coated with thin mercury film and SUOX cross-linked with gelatine | CV 0 to -0.75V | 12 |

to develop the ability to detect not only free sulphite but either total or bound sulphite so the entire fate of the added $SO_2$ is known. Recent work highlights a chemical approach to this problem which was investigated in this program.

Discussions between Professor Joe Shapter (Flinders University) and Dr Paul Smith (AWRI) led to an Honours project in 2010 focussed on developing a sulphite biosensor by using nanotechnology-based approach to surface-immobilising an enzyme capable of binding and detecting sulphite. The student has had some preliminary success in binding the enzyme and getting a response from it after exposing it to sulphite solutions. However in order to progress the research, resources beyond an honours student were required. This next stage of work was the subject of this project.

To achieve this final goal of a readily useable sensor that could be deployed in wineries, several experimental hurdles had to be addressed during the project. The routine construction of the sensor is a must and has to be followed by testing to assess the sensor's reproducibility, robustness, optimal storage conditions, lifetime, optimal operating conditions etc. It is likely to be necessary that such testing would have to occur on an on-going basis in the field and hence be readily doable by non-trained staff. Additional work is required to test the number of samples that can be reliably measured with one sensor and if any degradation occurs during the lifetime of the sensor. Such degradation could be addressed by the use of standards in the solutions tested meaning an individual sensor could be used for many tests but this again required work in the field and hence must be straightforward.

The effect of sensor exposure to the complex wine matrix on the sensor must also be extensively explored and understood. This will ensure that sulphite in the matrix can be measured reliably and accurately and also provide insights into further construction approaches that might be used to prolong sensor life.

**Flinders Capability**

The Smart Surfaces group in the School of Chemical and Physical Sciences at Flinders University had all the equipment and support personnel in place to carry out research program in the area of surface modification and characterisation. The school had a strong analytical chemistry group which can be involved to assess the validity of the analytical approaches. All

the equipment was already in place and in fact in most cases duplicated at least once at Flinders so any equipment malfunctions/breakdowns are manageable at Flinders. The AWRI will also be able to support the project with equipment access in the event of any unforeseen problems.

The chemicals required for the project were in house or readily available although more will be required during the project. The enzyme required is expensive and has a long delivery time. There are commercial suppliers but work to purify and test the production of enzyme is well worth exploring although very challenging. Most of the other chemicals are available throughout the school and any shortages can be addressed using material from other projects with the Smart Structures group or from other groups in the school.

Ready access to wine and beverage samples for testing were provided AWRI. These supplies are ideal as the sulphite content is known and hence they provide a rigorous test of the sensor under development. Other wine samples, if required, can be readily available through retail outlets.

## Outputs and Activities

### 4. Project Aims and Performance targets:

Milestones and Reports

| | |
|---|---|
| Output | Research Associate in Place |
| Target Date | 30/09/2011 |
| Activity | Hire Staff |
| Status | Achieved |

Dr. Cameron Shearer was hired in late November 2011 and worked full time on the project until February 2012. Dr. Renee Goreham took over on the project and work on the project continued uninterrupted. Additionally, Dr. Vanessa Thompson was also employed with other funding to undertake work on the project.

| | |
|---|---|
| Output | Trial Sensor Built/Tested |
| Target Date | 30/11/2011 |
| Activity | Gold substrates made, surface modifications done and tested, lifetime and storage experiments done, characterisation experiments complete |
| Status | Achieved |

The covalent attachment of enzyme for detection of sulphite was successful. The sensors seem to be stable over many weeks when stored in the fridge. Little degradation is observed over this time period. A second architecture to lower biofouling was tested but was not active.

| | |
|---|---|
| Output | Analytical Properties Tested |
| Target Date | 28/02/2012 |
| Activity | Calibration curves of standard solutions established, reproducibility tested, possible interferents identified |
| Status | Achieved |

Linear calibration curves were obtained using two different electrochemical analytical techniques: CV and chronoamperometery.

| | |
|---|---|
| Output | Wine Samples Test |
| Target Date | 30/05/2012 |
| Activity | Calibration curves of wine established, reproducibility tested, development of wine testing protocols |
| Status | On going |

Analysis of real wine samples lead to a suppression of the sulphite oxidation peak. Attempts to minimize the blocking effect of wine were completed but were only successful in slowing the blocking of the biosensor. Work is continuing in this area with the help of the AWRI.

| | |
|---|---|
| Output | Purification of Sulphite Oxidase |
| Target Date | 31/12/2013 |
| Activity | Purification of Protein from bacteria sourced from Scotland |
| Status | On going |

The enzyme was to be overexpressed in house to attempt to isolate active enzyme. It was planned to overexpress SUOX in *Escherichia coli* TP1000 from the Palmer Laboratory in the University of Dundee, Scotland. Despite several attempts the active enzyme has still not been isolated.

## 5. Method:

The enzyme sulphite oxidase was used to oxidise any sulphite present. The sensor is based on an architecture developed at Flinders.[13] In short, a thiol monolayer is built on gold which can be further used to attach the enzyme sulphite oxidase to the substrate. The addition of cytochrome C as a co-factor allows the enzyme to react with sulphite as it would in the biological system. One of the advantages of this system is that the enzyme is close to the surface and hence reactions that occur at the enzyme can be readily monitored by measuring the current produced at certainly voltages which are characteristic of the sulphite reaction. The specificity achieved by using the enzyme means that any signals observed must be due to the presence of sulphite and also will mean minimal sample processing is required.

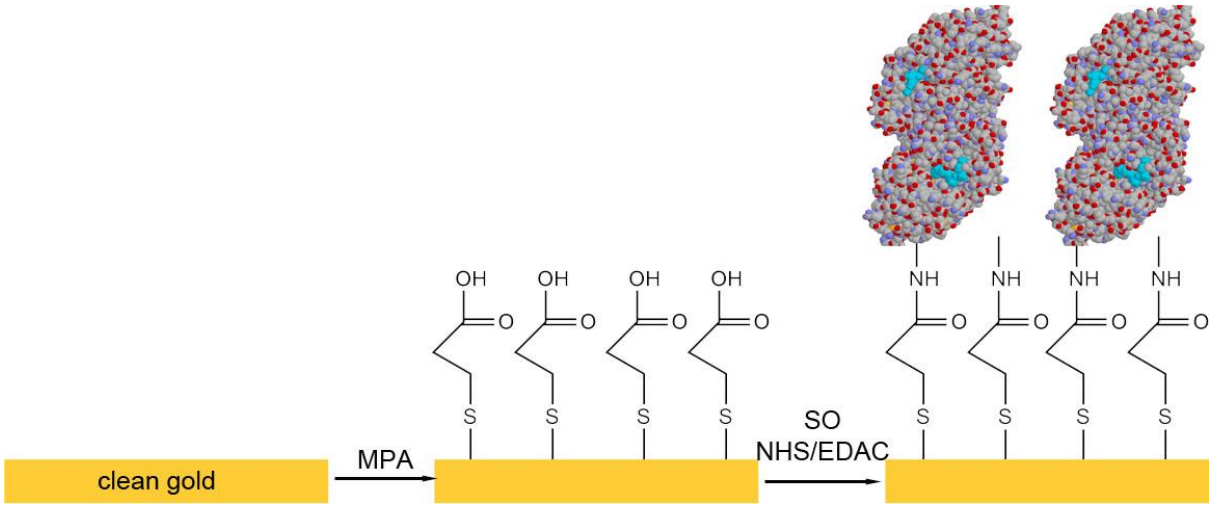
### Electrode Preparation:

Gold electrodes (2 mm diameter, CH instruments) were polished to a mirror finish on a polishing pad using alumina slurry, rinsed in $H_2O$, acetone, $H_2O$ and sonicated in $H_2O$ for 10 min to remove any remaining alumina particles. The gold electrodes were then cleaned electrochemically by completing CV scans from 0 – 1.2 V in 1 M $H_2SO_4$ until consistent reduction/oxidation peaks were observed (generally 5 – 10 sweeps).

### Biosensor preparation

*Mercaptosuccinic Acid Monolayer*

The biosensor was fabricated via a covalent linkage of sulphite oxidase (Monoclonal Anti-SUOX, produced in mouse) to a gold electrode following the basic method described by Losic, D. et al. (Ph. D. Thesis). The attachment method is shown schematically in Scheme 1.

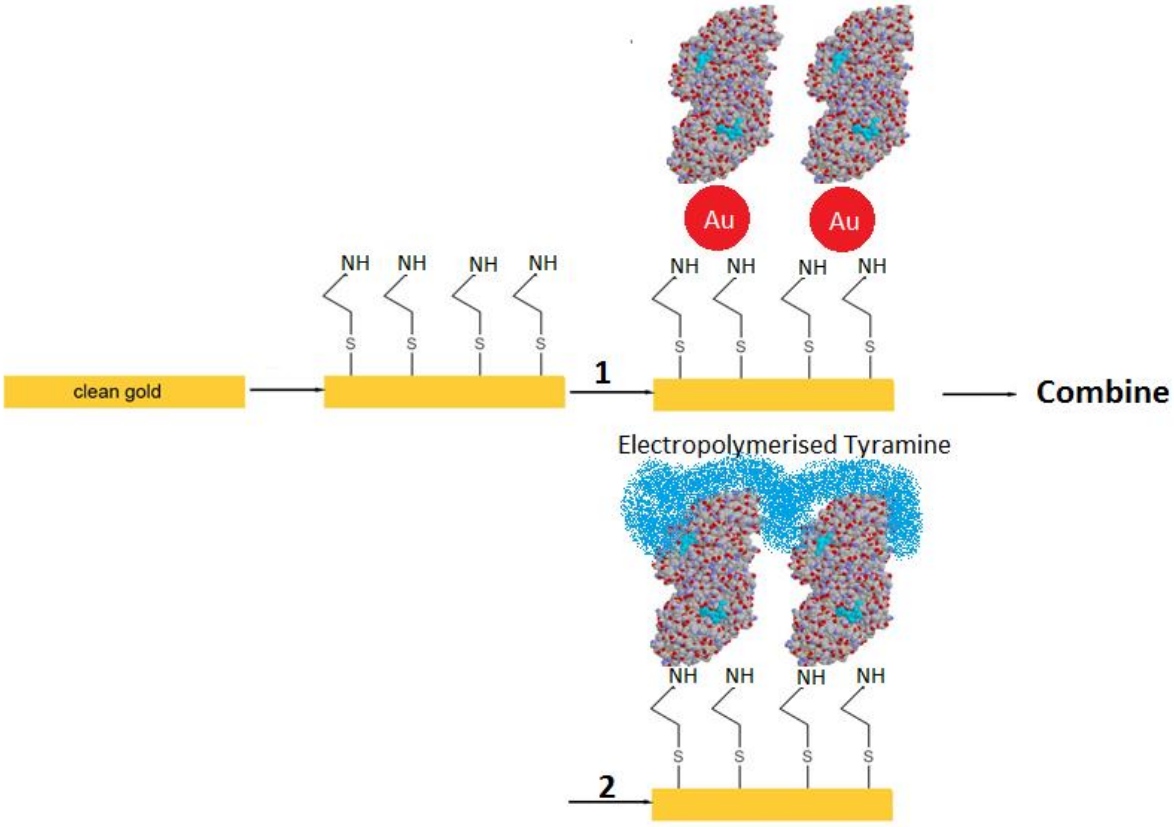



**Scheme 1: Schematic of covalent attachment of sulphite oxidase to gold electrode via activation of a MPA SAM.**

Prior to formation of mercaptopropionic acid (MPA) monolayer the electrodes were rinsed again in $H_2O$ and dried in a stream of nitrogen prior to immersing in a solution of 20 mM MPA in 75:25 ethanol (100 %):water for 24 hr in the dark.[10] The electrodes were then rinsed in 75:25 ethanol: water and dried under a stream of nitrogen then immediately immersed into a solution containing 0.002 M EDAC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride), 0.005 M NHS (N-hydroxysuccinimide) in pH 5.5 potassium phosphate buffer for 2 hr. This step acts to activate the carboxylic acid groups on the surface, promoting the subsequent reaction between SAM and enzyme. The electrodes were then rinsed gently with pH 5.5 buffer and immediately placed into enzyme solution (not dried). The solution for SUOX attachment consisted of 500 μg $L^{-1}$ SUOX in pH 7 potassium phosphate buffer, the enzyme attachment was allowed to continue for 90 min before the electrodes were removed, rinsed gently with pH 7 buffer and dried very gently with a nitrogen stream. Cytochrome-c (cyt-c) was adsorbed to the enzyme/SAM electrode by inverting the electrodes securely, and dropping ~10 μL (enough to completely cover electrode area) of 50 μM cyt-c (in pH 7 buffer) onto the electrode and allowing dried for 15 – 20 min.[14] Excess cyt-c was gently rinsed with pH 7 buffer, the electrodes were kept in the fridge, in the dark in pH 7 buffer whenever not in use.

*Cystemamine Monolayer*

The as prepared gold electrodes were immersed into 0.2 mM solutions of cysteamine (Sigma Aldrich) for 24 hours




**Scheme 2: Schematic of modified biosensor formation using 1: gold nanoparticles and 2: electropolymerised tyramine.**

**Electrochemical Analysis of Biosensor**

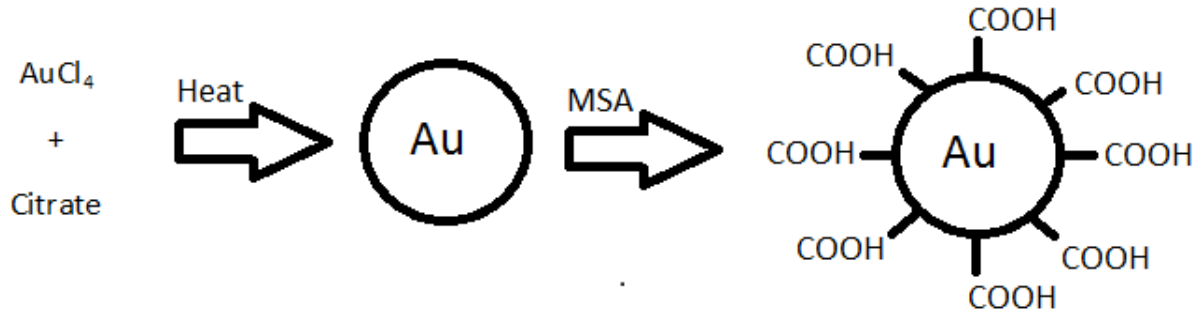
Electrochemistry used the CH electrochemical analyser with a standard 3 electrode system, working: biosensor, reference: Ag/AgCl (saturated), counter: platinum wire. Electrochemistry was completed by beginning with a known volume of pH 7 phosphate buffer and adding known volumes of either sodium sulphite stock (0.1 M) or wine. Cyclic voltammetry was typically done by scanning from -0.1 – 0.8 V, however, the maximum potential was occasionally increased to 1.2 V, this removed adsorbed particulates that reduced biosensor response and was typically completed after wine analysis. Chronoamperometry was completed at a potential of 0.4 V.

**Preparing Surfaces of Self Assembled Monolayers and gold nanoparticles for AFM Imaging**

Initially gold surfaces were prepared using evaporated gold but yield rough surfaces, thus the sputter coater was used. Silica surfaces were prepared by cleaning in freshly prepared piranha solution, rinsed with Milli-Q water and ethanol. The cleaned silica wafers (10 x 10 mm) were coated with 5nm of titanium and 140 nm of gold using the Quorumtech K757X Sputter coater.

**Gold Nanoparticle Synthesis**

Gold nanoparticles of were synthesised by citrate reduction of $HAuCl_4$, where 16 nm diameter were synthesised from 50 mL of a 0.01 % boiling solution of $HAuCl_4$, to which 1 mL of a 1% solution of sodium citrate was added under vigorous stirring. The solution was left to boil for 20 min and then allowed to cool to ambient temperature.[15] The AuNPs were then surface modified with 2-mercaptosuccinic acid as in Zhu et al.[16] and the particle diameter was confirmed via AFM images (also see Scheme 2: pathway 1).[17]




**Scheme 3: Gold nanoparticle synthesis via citrate reduction of gold chloride.**

**Electropolymerised Tyramine**

Tyramine was electropolymerised onto the prepared gold (Au) surface (i.e. gold electrode or as prepared above), as done in Losic et al.[18] A solution of 0.1 M of Tyramine hydrochloride (sigma Aldrich) in pH 7 Phosphate Buffer solution (PBS) was made. The applied potential initially used during polymerisation was between 0 and +1.3 V versus Ag/AgCl with a scan rate of 0.5 V/s. The numbers of scans were varied from 0-30. Surface topography was analysed using AFM (also see Scheme 2: pathway2).

**Enzyme Assay**

To establish a usable amount of enzyme to immobilise on the surface of the biosensor, a method for enzyme activity needed to be conducted. Shown in Table 2 is a summary of the methods used after purification of sulphite oxidase.

**Table 2: Summary of the various methods used to detect sulphite oxidase activity**

| Assay | Derived SUOX | Method | Reference |
|---|---|---|---|
| Activity of SUOX in Soil | In Soil | Enzyme activity was determined spectrophotometrically by measuring the disappearance of the yellow colour of $K_3Fe(CN)_6$ due to the reduction of $Fe^{3+}$ at 420 nm | 19 |
| Direct Spectrophotometric Assay to detect sulphite to sulphate reaction | SUOX purified from rat liver | Direct decrease in absorption of Sulphite in the short UV range (212 nm) was measured as Sulphate has no absorption. | 20-22 |
| Indirect Spectrophotometric Assay to detect the reduction of Cytochrome c | Homo sapiens Recombinant SUOX | Monitoring the reduction of Cytochrome c at 550 nm | 21,23,24 |
| Indirect Spectrophotometric Assay to detect the reduction of ferricyanide | Homo sapiens Recombinant SUOX | Monitoring the reduction of Cytochrome c at 550 nm-activity of the Molybdenum was measured by monitoring the reaction of ferricyanide at 420 nm | 21,25 |

**Indirect Spectrophotometric Assay to Detect the Reduction of Cytochrome c**

The reduction of cytochrome c in the presence of sulphite oxidase and sulphite was monitored to determine the activity.[21,23,24] For assays, cuvettes were filled with 0.1 M Tris-HCl, pH 8.5, with 0.1 mM of EDTA, 50 µL of 1 mM cytochrome c and purified enzyme (concentration varies) in a final volume of 1 mL. The rate of increase in absorbance at 550 nm was measured before and after the addition of 8 µL of 0.1 M sodium sulphite. One unit of sulphite oxidase activity is defined as the amount of enzyme required to yield an absorbance change of 1.0/min at 25℃. Under these conditions one unit of sulphite oxidase activity corresponds to the reduction of 0.053 µmol of cytochrome c/min.[24]

**Indirect Spectrophotometric Assay to Detect the Reduction of Ferricyanide**

Enzyme was added to cuvettes containing 4 x $10^{-4}$ M sodium sulphite, 4 x $10^{-4}$ M potassium ferricyanide, and 1 x $10^{-4}$ M EDTA buffered at pH 8.5 with 0.10 M Tris-HCl. The reaction volume was 2 mL and the temperature was at room temperature. The reduction of ferricyanide was followed in terms of a decrease in absorbance at 420 nm.

More details can be found in the lab book of Cameron Shearer/Renee Goreham, sulphite biosensor including material location and supplier (pg. 1), buffer preparation (pg. 14), mass calculations for MPA, EDAC, NHS, enzyme, cyt-c solutions (pg. 15), preparation of sulphite stock (pg. 17). Copies of these documents are readily avaibale upon request.

## 6. Results/Discussion:

Initially, the biosensors that were produced previously were reproduced and tested. Once similar signals were obtained, investigations on how to get the biosensors to work in wine solutions were trialled. It was then realised that the purchased sulphite oxidase enzyme was the antibody for sulphite oxidase and had no enzymatic activity. While another source was found, the immediate biosensor was modified using electropolymerised tyramine and gold nanoparticles. In addition to this work, the mechanism for the signal response was also investigated. Initially it was thought to be cytochrome c, which was shown to react with sulphite but further work established it could be a background signal response in the presence of the gold electrode and sulphite. In addition, a source of sulphite oxidase was found from OriGene, (Australian Biosearch) that had no enzyme activity with the cytochrome c but in the presence of suflite, had low enzyme activity allowing for the reduction of ferricyanide. This assay confirms that there was some molybdenum centre activity. Many methods have been established that could be implemented at a later date that would provide a successful biosensor to be developed.

Biosensors

Biosensors were prepared simultaneously (method described by Cameron Shearer and Jared Parsons) and tested using Cyclic Voltammetry (CV) by adding aliquots of sulphite to pH 7 buffer. Amperometry was used to calibrate each biosensor and was done by adding 50 µL aliquots of 0.01M sodium sulphite every 30 s to 10 mL of pH 7 Buffer over a total period of 1020 s. The average of the peak current over each 30 s interval was calculated and plotted (as shown in figures 1-3).

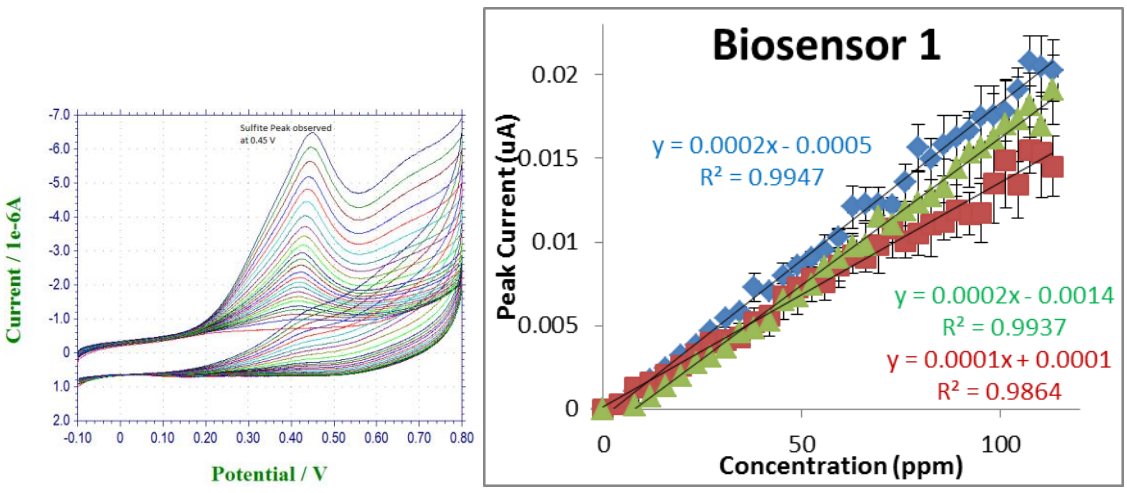



**Figure 1: Biosensor 1 CV results and the calibration produced via amperometry.**

Biosensor 1 showed linear curves and were somewhat reproducible. The biosensor was stored for more than 2 h prior to the first trial (blue) and third trail (green), whereas the second trial (red) was only stored in the fridge for an hour before use. This indicates that storing the biosensor in the fridge in pH 7 buffer enables any remaining sulphite ions present to de-absorb off the surface.

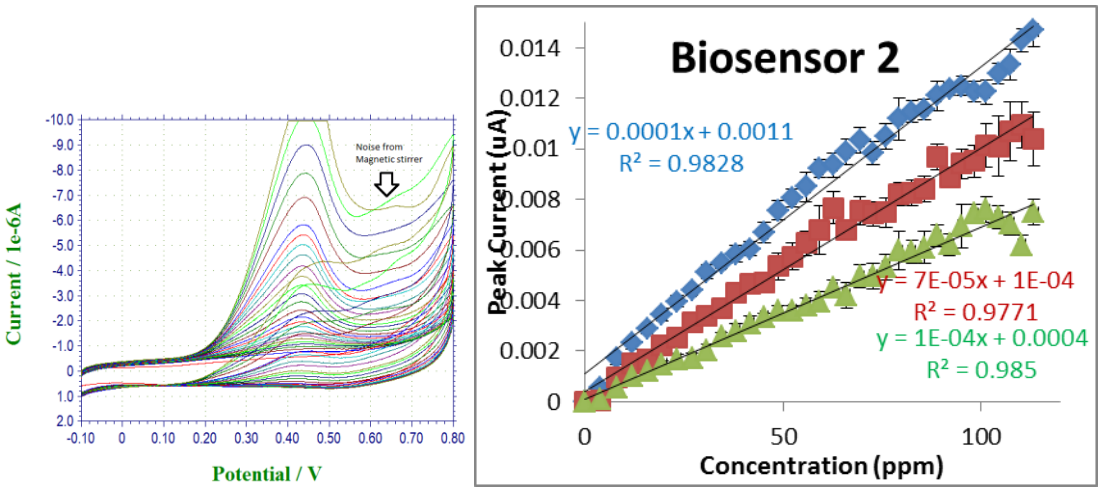



**Figure 2: Biosensor 2 CV results and the calibration produced via amperometry.**

Biosensor 2 had a significant loss in sensitivity over the 2 days of use. The first and second trial (blue and red respectively) were both done within an hour and the third trial was done the following morning. All showed a linear fit, but also showed the least sulphite sensitivity.

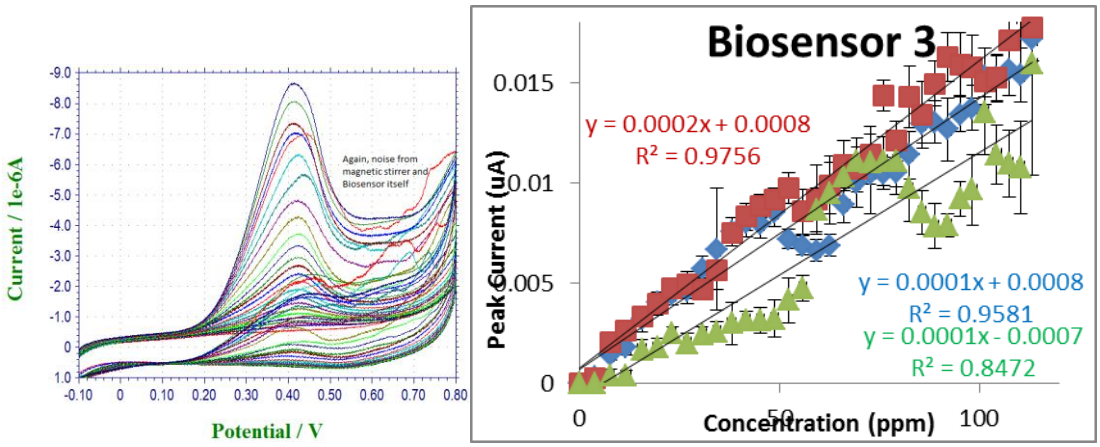



**Figure 3: Biosensor 3 CV results and the calibration produced via amperometry.**

Biosensor 3 was not as accurate and the previous two showing significant standard deviation. This would most likely be due to the preparation or the enzyme film or the gold surface pre-treatment. With that said, the overall slopes were linear and trials one and two (blue and red respectively) were quite similar. Similar to the biosensor 2, the first and second trial was done within an hour of each other whereas trial 3 was done early the following day.

Anti-Sulphite Oxidase instead of Sulphite Oxidase

It was found that the thought to be sulphite oxidase enzyme purchased from Sigma Aldrich was an antibody (as per the name, Monoclonal Anti-SUOX, antibody produced in mouse purified immunoglobulin). As Sigma-Aldrich has not supplied sulphite oxidase since 2004, another source needed to be obtained. OriGene was found to supply a sulphite oxidase protein (Purified recombinant protein of Homo sapiens sulphite oxidase (SUOX), nuclear gene encoding mitochondrial protein, transcript variant 1) and was purchased immediately.

Although the incorrect protein was used for the biosensor, the biosensor provided a signal with increasing sulphite concentration that appeared at 0.4V and was thought to be the oxidation of sulphite to sulphate. With that said, there was no reduction peak representation the reduction of sulphate back to sulphite (expected if enzyme was present). It could have been possible that the cytochrome-c may aid in the sulphite oxidation so a biosensor with no anti-SUOX was trialled.[26]

Work done by Kalimuthu et al.[11] had showed that a low ratio of mediator/enzyme ratio showed a sigmoidal voltammogram, indicating a steady state process.[27] They found that once the amount of cyt c was increased (when compared to SUOX), the sulphite reaction was unable to supply a sufficient amount of electrons allowing for a steady state to be maintained for cyt c and resulted in a peak shape voltammogram (transient). The tailing off was thought to be an accumulation of oxidised cyt c in the diffusion layer. The biosensor produced in this work may be having a similar effect. It was also stated by Kalimuthu et al.[11] that high potentials (>+600 mV vs Ag/AgCl at pH 8) resulted in nonspecific oxidation of sulphite but the data for these results were not shown. With an excess amount of cyt c on the surface of the biosensor, the sulphite may be oxidised (either by the high current or the cyt c itself) and as there is no enzyme with the Mo centre to reverse the cyt c oxidation, no reduction peak of either sulphate or oxidised cyt c is observed. A combination in sulphite oxidation and the accumulation of cyt c oxidation may be what gives the signal seen in past work but more studies need to be done confirm this process.

Wine Analysis

Wine analysis using CV and was done by adding 1.5 mL of white wine to 10 mL of pH 7 buffer solution. The enzyme SUOX is active in the pH of 5-8 and as wine is acidic, 1.5 mL of wine was determined to be the maximum addition to maintain this range. As shown in Figure 4, when the wine was added (brown), the appeared to be a broad peak, which decreased with the further additions of sulphite. As hypothesised in previous work, there appears to be something in the wine blocking the biosensor.

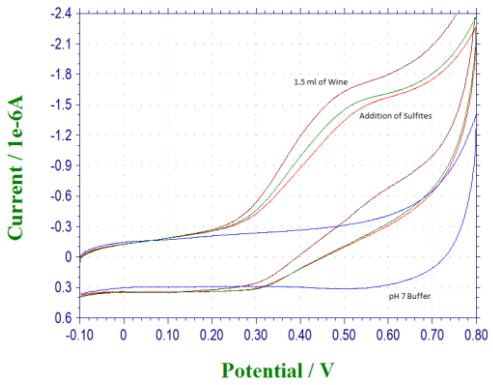



**Figure 4: Addition of white wine (1.5 mL), followed by additions of sulphite (first 500 µL, followed by 1 mL).**

The polyphenols in wine are large hydrophobic molecules and are predicted to be blocking the biosensors and preventing them from working. To determine if this is true, 1.5 mL of white wine stripped of polyphenols was added to 10 mL of pH 7 buffer solution (as done

above). As shown in Figure 5, a similar effect was shown where there was no observable sulphite peak, indicating that there was still something interacting with the biosensor preventing the sulphite peak to be seen.

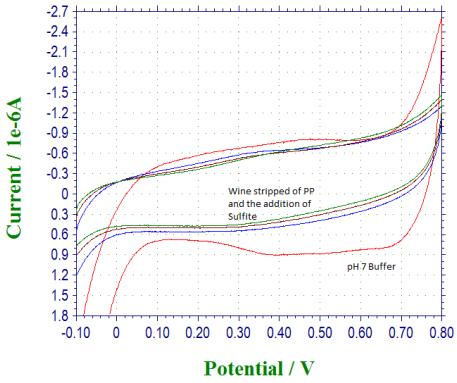



**Figure 5: Addition of white wine stripped of polyphenols (PP), followed by additions of sulphite (first 500 µL, followed by 1 mL).**

To determine whether the polyphenols were contributing or not to the inefficiency of the biosensor, CV scans were done on

1 10 mL pH 7 buffer
2 an aliquot of 500 µL of sulphite
3 another aliquot of 1 mL of sulphite
4 followed by the addition of 0.1 mL of concentrated polyphenol isolate.

As shown in Figure 6, there was still a decrease of the sulphite peak as a result of the introduced polyphenol.

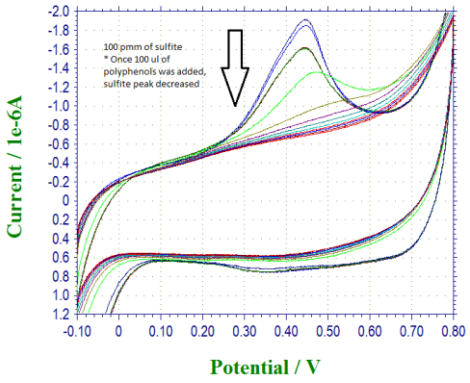



**Figure 6: An addition of concentrated polyphenol isolate added to 10 mL pH 7 buffer solution containing 100 ppm (1.5 mL) of sulphite solution.**

As the biosensors were inactive in the wine stripped of polyphenols, there is something else that is contributing. To determine whether proteins are at fault or not to the inefficiency of the biosensor, CV scans were done on

1 10 mL pH 7 buffer
2 an aliquot of 500 µL of sulphite
3 another aliquot of 1 mL of sulphite

4 followed by the addition of 100 mg/L of grape protein

Again, there is decrease in the sulphite peak as a result of the proteins in solution, indicating that both proteins and polyphenols are causing the biosensors to become inactive. The next question was what constituents of wine were interfering, and how can the biosensor be modified to prevent this problem.

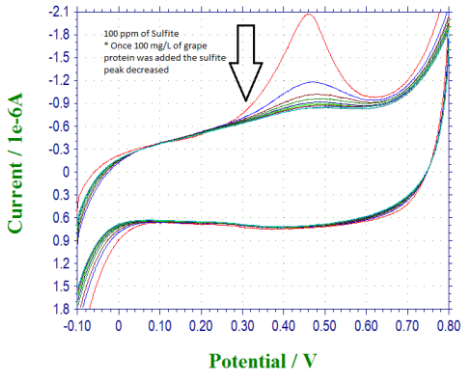



**Figure 7: Addition of 100 mg/L of grape protein added to 10 mL pH 7 buffer solution containing 100 ppm (1.5 mL) of sulphite solution.**

NOTE: The CV scans in Figure 7 were done on Biosensor 2 in one day and to indoor to get the biosensors working again, CV scans up to 1.2 V were done to oxidise some material, hence unblocking the electron pathway.

### Modifying the Biosensor

### Electropolymerisation of Tyramine

Silica surfaces were coated by gold (140 nm) using a sputter coater. Tyramine was electropolymerised onto the prepared gold (Au) surface, as done by Losic et al.[18] A solution of 0.1 M of Tyramine hydrochloride in pH 7 Phosphate Buffer solution (PBS) was made. The applied potential initially used during polymerisation was between 0 and +1.3 V versus Ag/AgCl with a scan rate of 0.5 V/s. The numbers of scans were varied from 5, 10 and 25. The scans are shown graphically in Figures 8 -10.

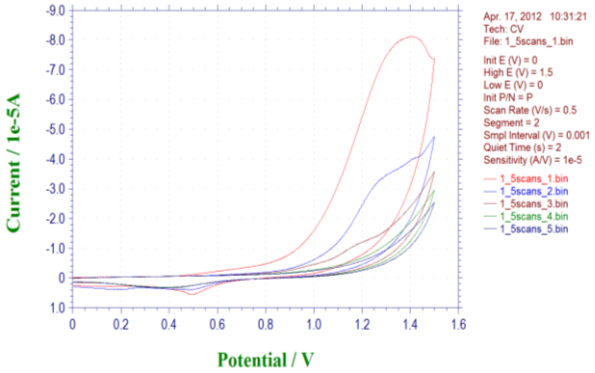



**Figure 8: 0.1 M of tyramine electropolymerised using 5 scans.**

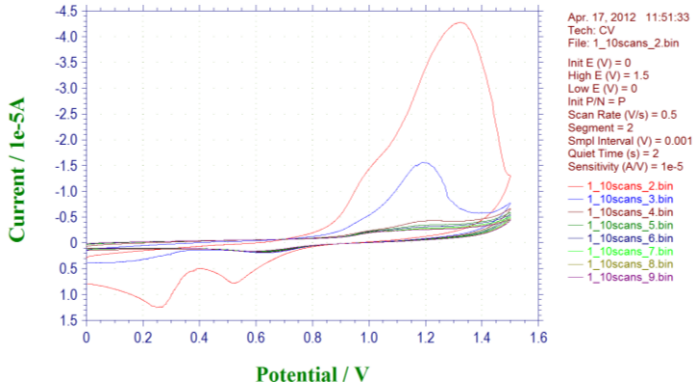



**Figure 9: 0.1 M of tyramine electropolymerised using 10 scans.**

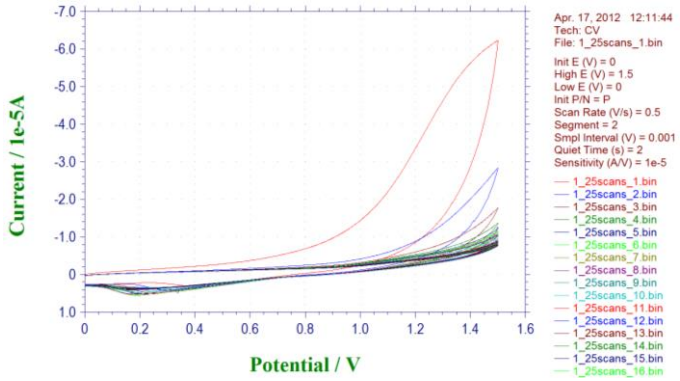



**Figure 10: 0.1 M of tyramine electropolymerised using 25 scans.**

It appears that the polytyramine has been deposited as the scans show a decrease in electrode conductance, which is expected as a tyramine is insulating. The gold surface appears very rough but the evaporation process is supposed to yield a surface roughness of less than 5 nm. In addition, tapping mode would provide a more accurate image as the tip with contact mode got "stuck" in the surface. Once these problems have been addressed, the enzymes can be introduced into the process.

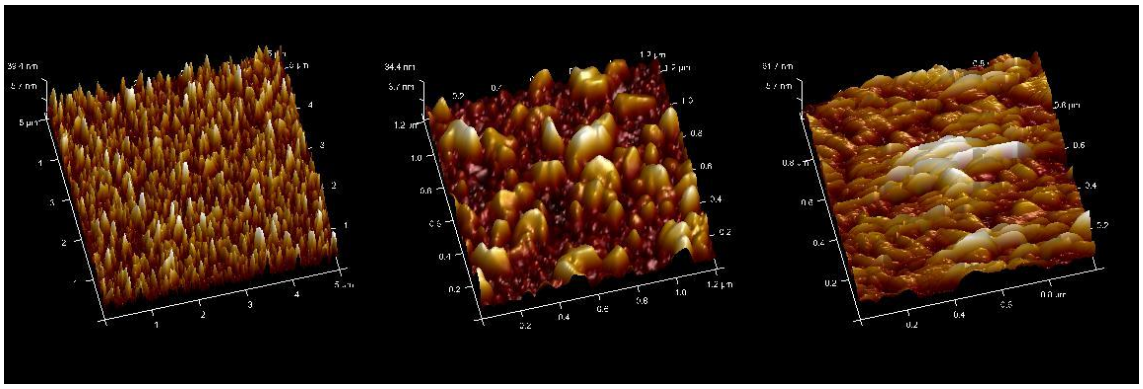


**Figure 11: AFM image of evaporated Au, a cropped image (1.2x 1.2um) and electropolymerised tyramine after 5 scans. NOTE: the Au is a 5x5 um image and the polytyramine is 1.2x1.2 um.**

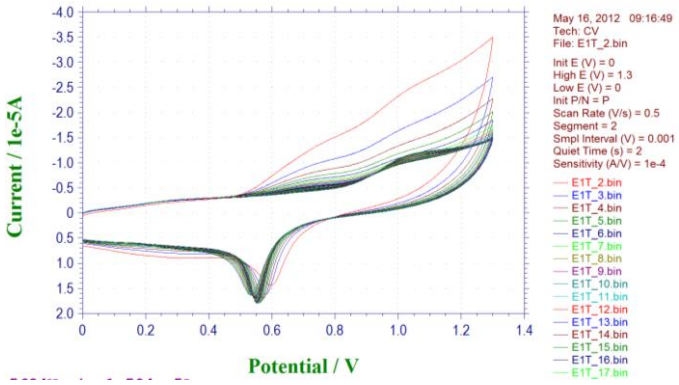



**Figure 12: 30 scans of electropolymerised 0.1 M tyramine with SOUX and cyt-c on a gold electrode**

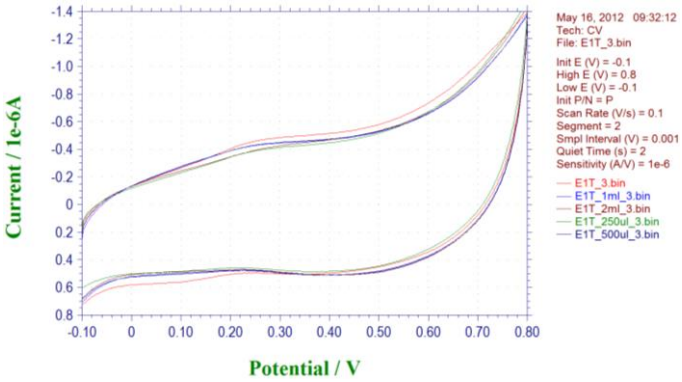



**Figure 13: Testing the modified biosensor with sulphite additions to PBS pH 7.**

Using a 3 mm Au electrode (as the 2 mm was unpredictable and didn't always work), 0.1 M tyramine hydrochloride was deposited using 5, 15 and 30 scans (see Figures 15 - 19). The enzymes were added and AFM was used to see if there was any change in surface topography as a result of the added proteins that may confirm the incorporation of proteins in the film.

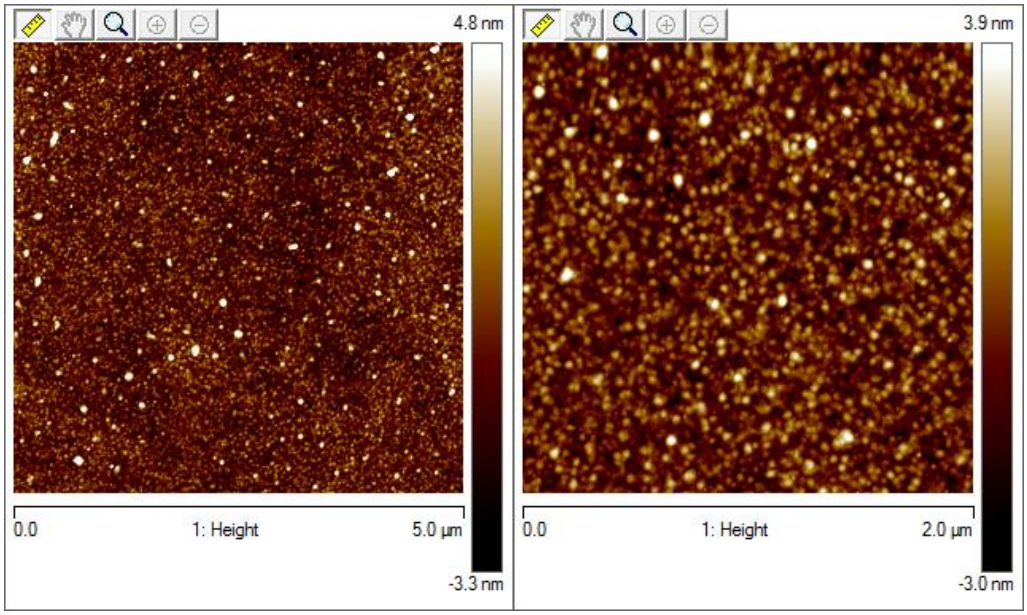



**Figure 14: AFM images of flat Au using sputter coater**

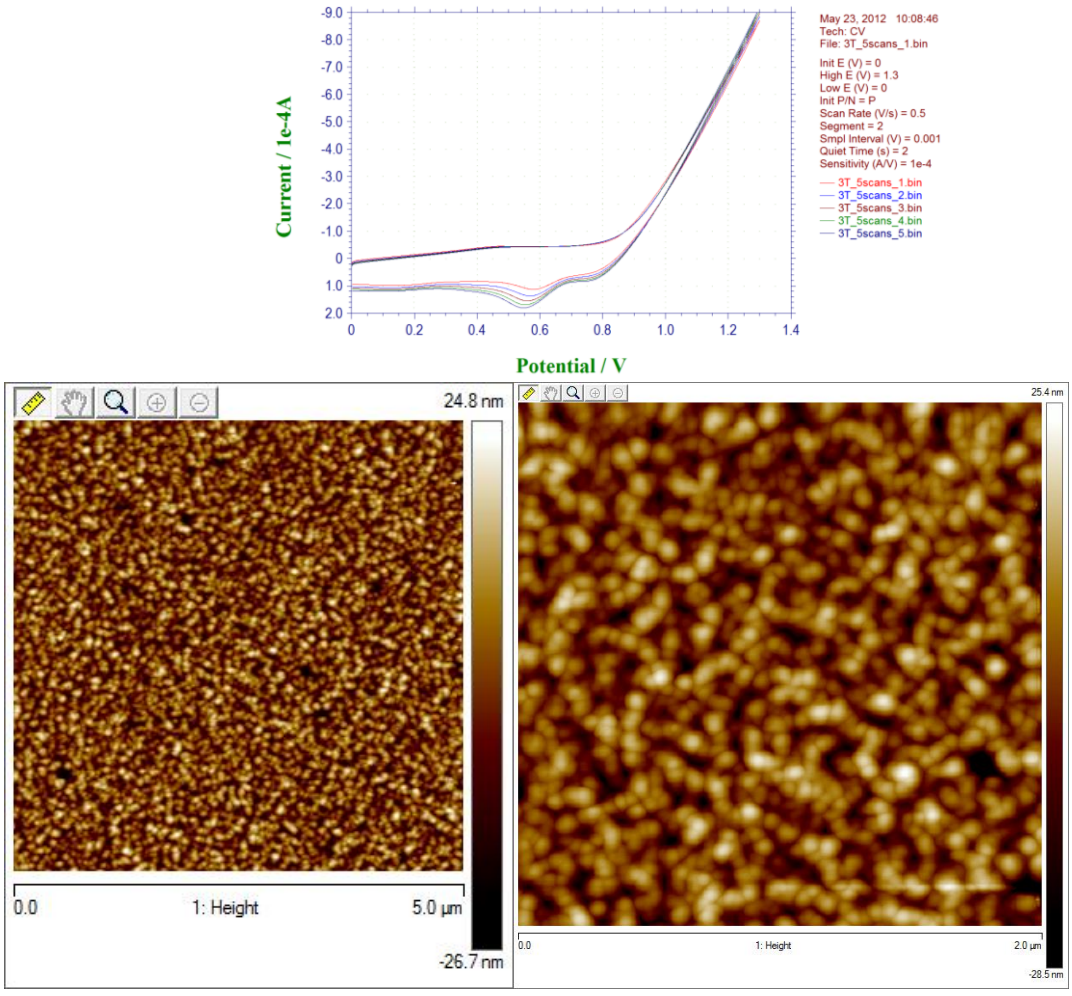



**Figure 15: AFM images of electropolymerised tyramine (5 scans) on Au.**

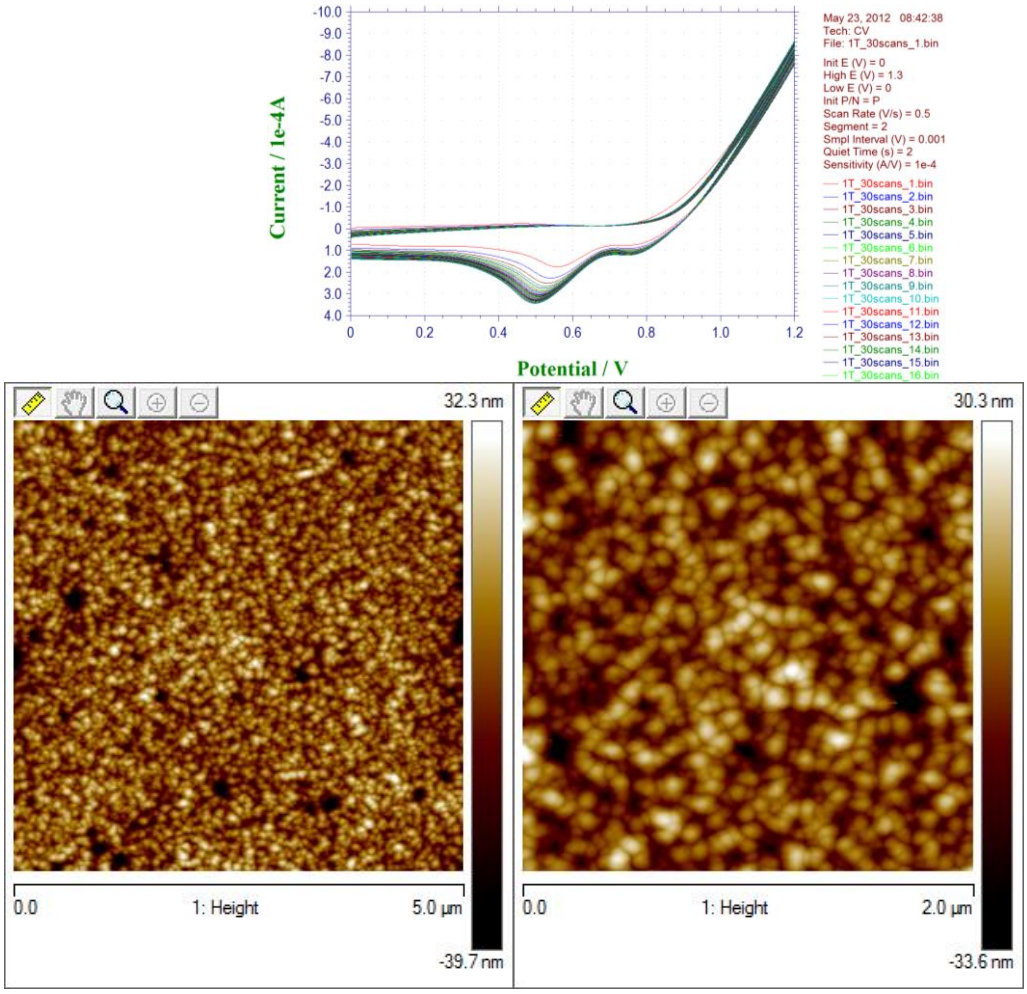



**Figure 16: AFM images of electropolymerised tyramine (30 scans) on Au.**

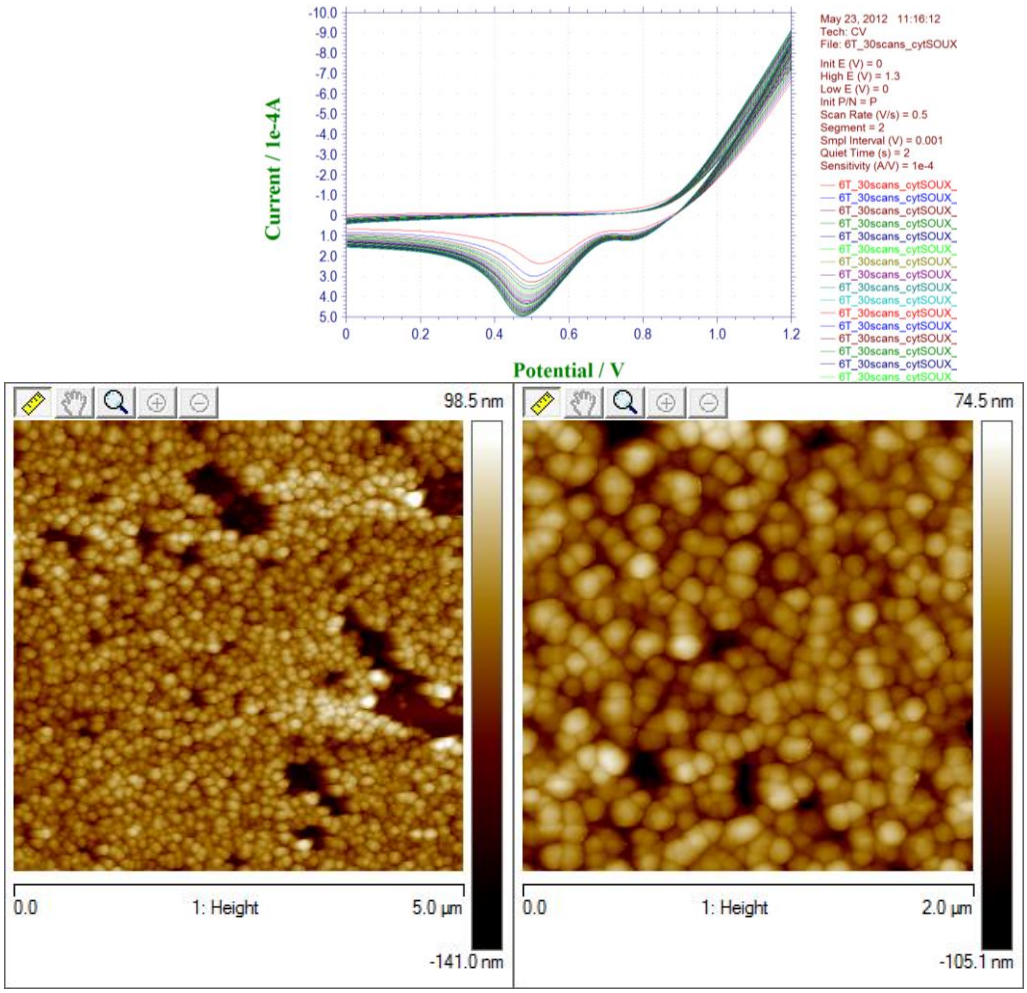



**Figure 17: AFM images of electropolymerised tyramine (30 scans) with SUOX and cyt-c on Au.**

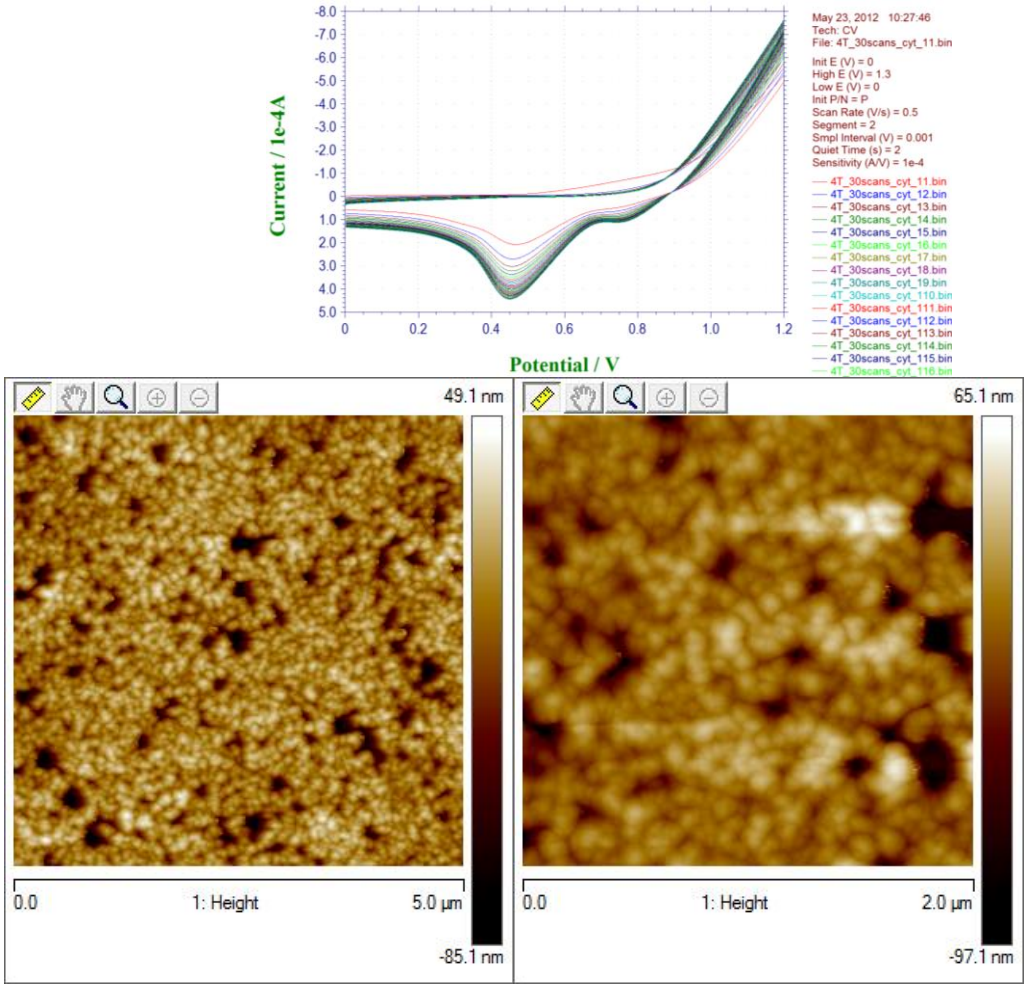



**Figure 18: AFM images of electropolymerised tyramine (30 scans) with cyt-c on Au.**

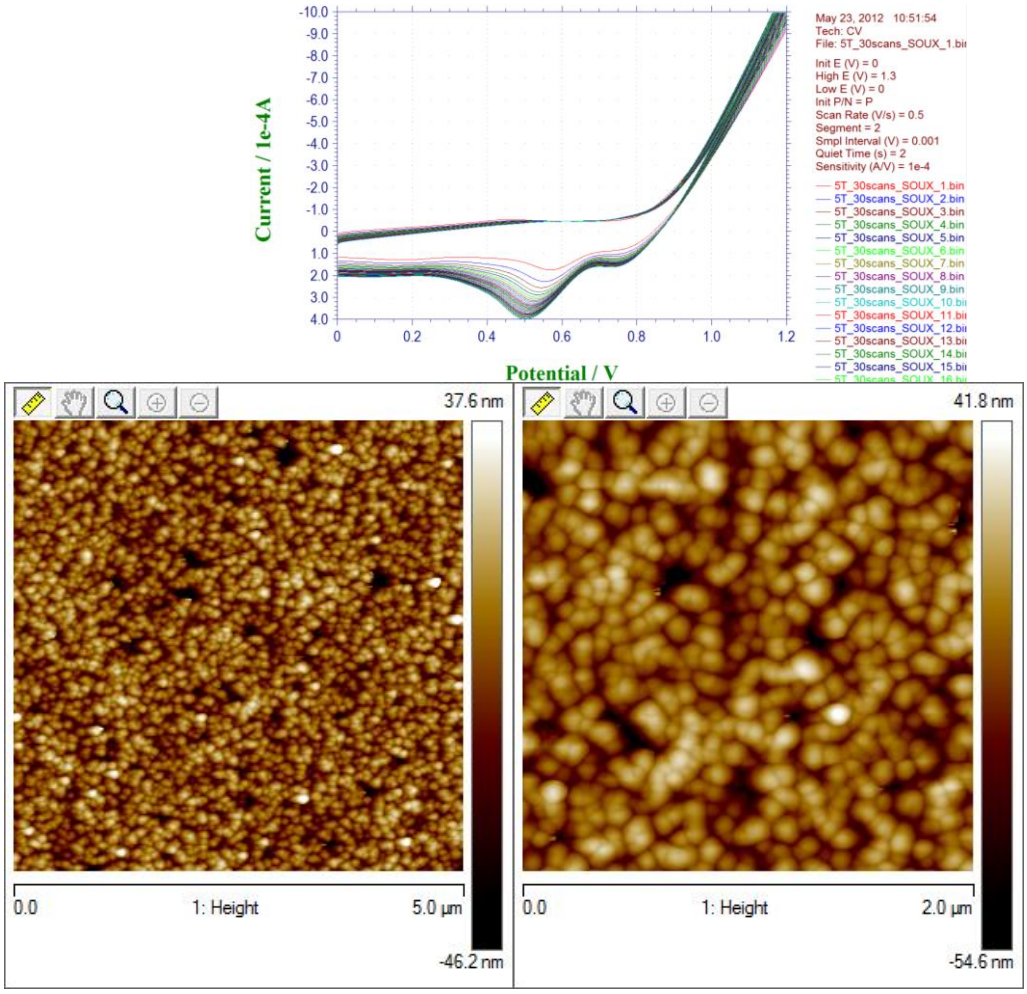



**Figure 19: AFM images of electropolymerised tyramine (30 scans) with SUOX on Au.**

The biosensors were made as previously described and calibrated (see Figure 20 and Figure 21) and biosensor 3 was used to determine the effect of electropolymerised tyramine deposited using 1 cyclic scan. As can be seen (see biosensor 3-1 T in Figure 21), there was little effect on the biosensors calibration curve. Increased scans will be later used to determine if there is a threshold thickness before the biosensor becomes inactive. Although the biosensor with 1T worked in the model solutions containing sulphite, once placed in a wine solution, a similar effect was seen where the biosensor become inactive (Figure 22).

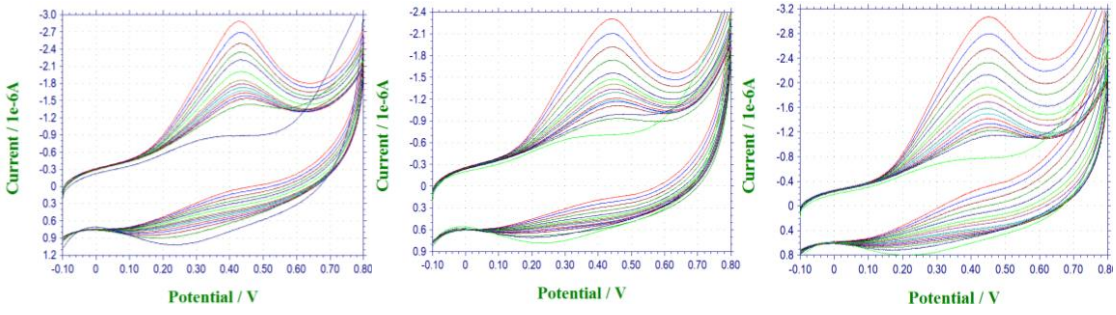



**Figure 20: The cyclic voltammograms of the freshly prepared biosensors.**

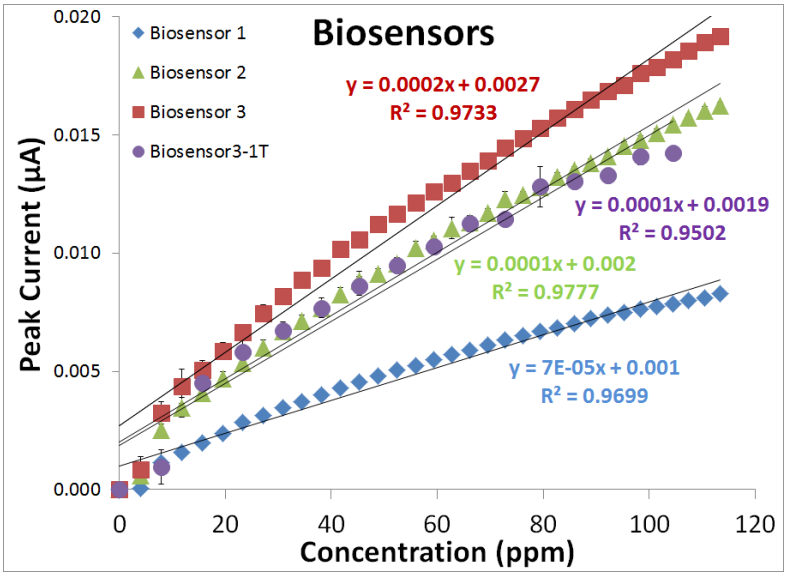



**Figure 21: Calibration curves for biosensors 1 to 3 and biosensor 3 with electropolymerised tyramine deposited with 1 scan.**

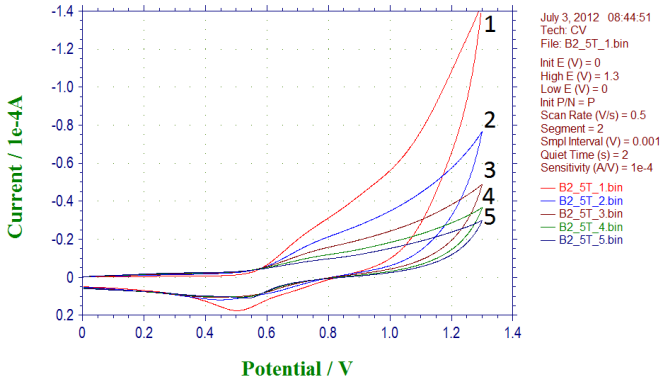



**Figure 22: Electropolymerised tyramine on a prepared biosensor using 5 scans of cyclic voltammetry.**

An electropolymerised layer of 0.1 M tyramine was deposited on a working biosensor (prepared as described earlier) using 5 cyclic voltammetry scans. As seen in the voltammograms (Figure 22), the scans show the deposition of the thin film as it becomes more insulating, hence the decreasing current. The modified biosensor was then tested for effectiveness (Figure 23) and proved to still detect the sulphite levels in PBS solution but once a wine aliquot was added (750 µL in 5 mL of spiked PBS) the biosensor became inactive, as seen in previous trials. This may be due to the adsorption of polyphenols and proteins to the biosensor, hence inhibiting the electron transfer or the sulphite in solution is unable to be oxidised in the presence of these macromolecules. It may be prevented once the sulphite oxidase replaces the Anti-SUOX protein.

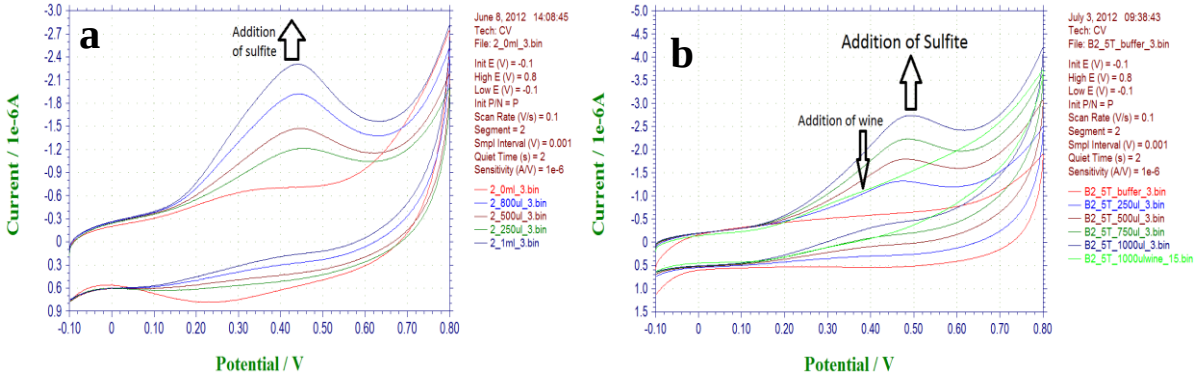



**Figure 23: A biosensor without (a) and with (b) electropolymerised layer of tyramine (5 scans) used to detect sulphite in PBS (250 µL aliquot additions) and with the addition of wine (fluorescent green).**

### Removing the Anti-SUOX step

To test whether the signal is a result of non-specific sulphite oxidation and/or cyt c oxidation, a biosensor was made as done prior but removing the Anti-SUOX layer from the electrode. As can be seen by the CV below, once the 250 µL aliquots were added, a similar effect was seen where a peak representing either the oxidation of sulphite and/or cyt c.

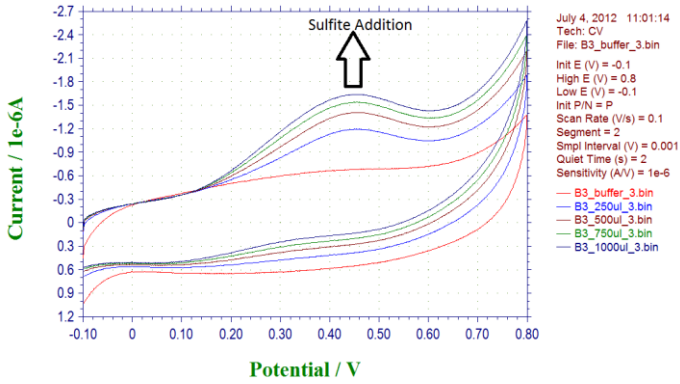



**Figure 24: CV scans of biosensor without Anti-SUOX layer and sulphite (250 µL aliquot) additions.**

### Dialysis Membranes

Dialysis membranes with different molecular thresholds (MW 10000, 5000, 2000 and 1000) were used on the already made biosensors. The dialysis membranes were placed over biosensor electrode to attempt to filter out substituents of wine that may be interfering with enzymatic activity.

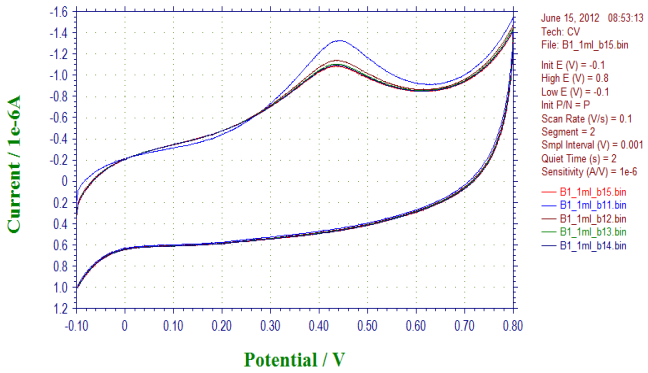



**Figure 25: CV scans of biosensor with 10000 MW a dialysis membrane.**

Similar to previous work, the first scan (blue) was much better than the subsequent scans. This was also noticed in an earlier experiment when the solution was not stirred. In that case it was determined that enzymatic activity lowered the local concentration of sulphite around the electrode, when stirring was applied the sulphite concentration was kept constant throughout the solution. Here we have a dialysis membrane, restricting flow of solute between the solution and the electrode.

When trialled with 5000 and 2000 MW dialysis membranes the current was completely blocked and no current was observed.

### Incorporating Gold Nanoparticles into Biosensor

Gold nanoparticles were synthesised by citrate reduction of gold chloride (see Scheme 3) and surface modified with mercaptosuccinic acid to provide more stable gold nanoparticles with a functionalised surface of carboxylic acid groups. The negatively charged carboxylic acid groups electrostatically bound to the self-assembled monolayer of cysteamine that provided a functionalised surface of positively charged amine groups. It was thought that the gold nanoparticles would increase the amount of absorbed protein (for example cytochrome c) by increasing the surface area.[10,28] Although there appeared to be a slightly higher signal response (Figure 27), this was not able to be replicated.

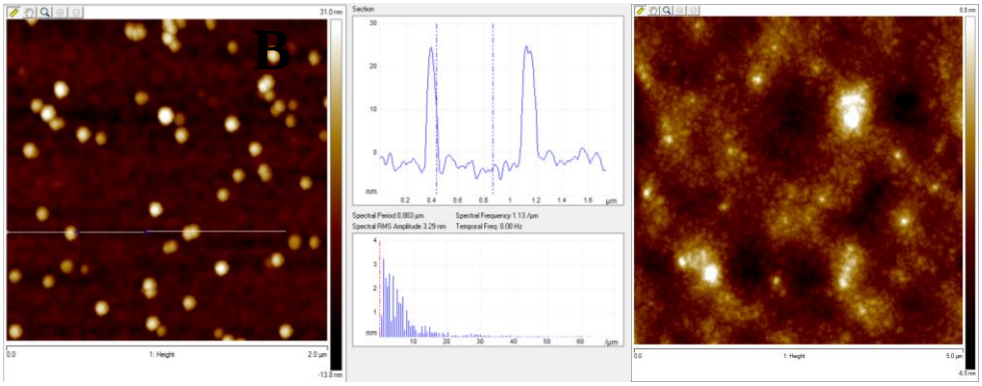



**Figure 26: Gold surface with a cysteamine self-assembled monolayer with A) gold nanoparticle immobilised onto surface, B) verification of the size and C) gold nanoparticles with cytochrome c absorbed to the surface.**

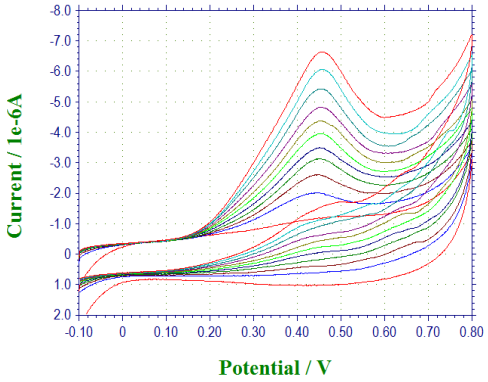



**Figure 27: Cyclic voltammogram of biosensor with cytochrome c immobilised on to a surface covered in gold nanoparticles immobilised**

**Enzyme Activity of Sulphite Oxidase Purchased from OriGene**

**Indirect Spectrophotometric Assay to Detect the Reduction of Cytochrome c**

The activity of sulphite oxidase was tested to obtain a unit of activity that can then enable a known amount of active enzyme that can be immobilised onto the surface of the biosensor (and an appropriate ratio between cytochrome c can be established. The first assay trialled observe the reduction of cytochrome c in the presence of sulphite oxidase and sulphite.[21,23,24]

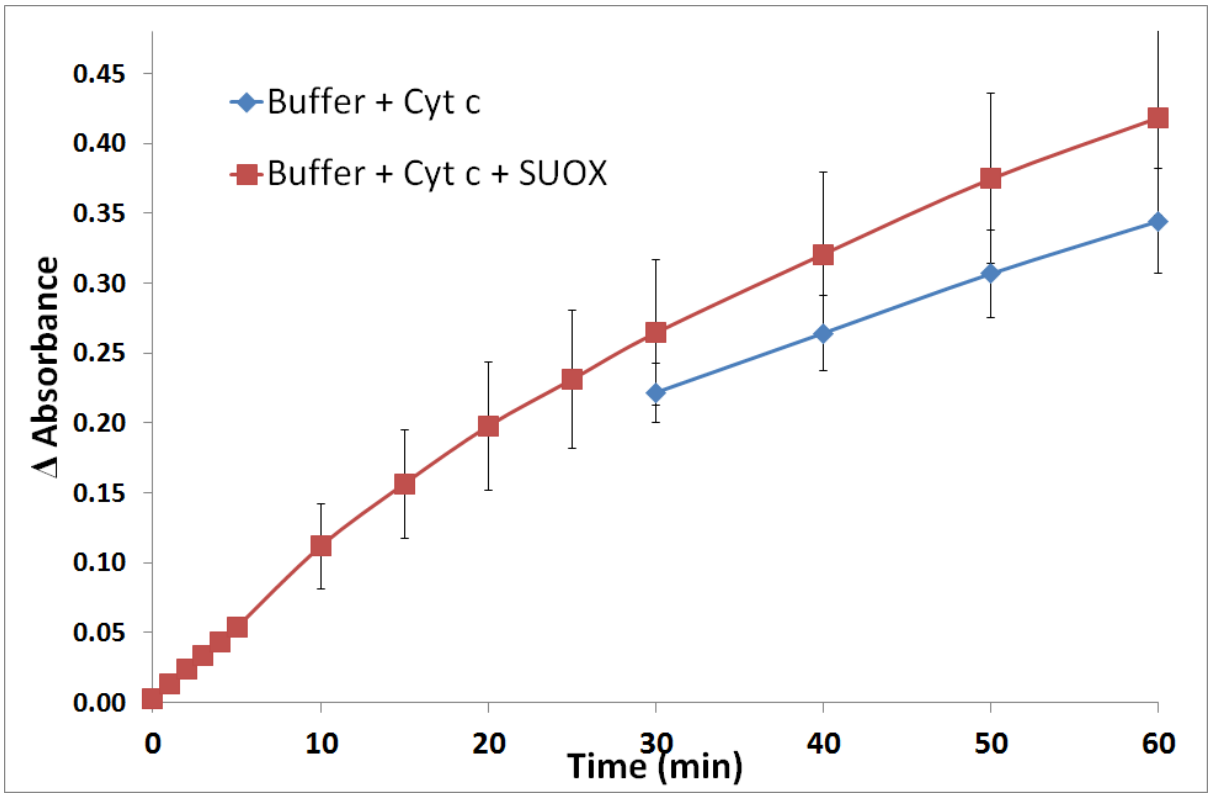



**Figure 28: Absorbance at 550 nm relating to the reduction of cytochrome c after the addition of 40 µL of 0.1 M Sodium Sulphite.**

It should be noted that the supplied SUOX was of low concentration (0.02 mg/mL before dilution in 2 mL) when comparing to concentrations in the literature (3-50 mg/mL). This may be the reasoning behind the low increase in activity when compared to the increase with the addition of sulphite and no SUOX. It was shown that there was no statistical difference between samples with or without SUOX (ANOVA, p=0.8739). It is thought that increasing the concentration SUOX and/or sulphite, the assay will be improved and activity can be determined. This will be tested in the coming weeks, once new SUOX arrives.

The concentration of the sulphite oxidase was increased to 0.14 mg/mL (before dilution in 2 mL) and the sodium sulphite was increased to 1 M solution to see if any enzyme activity present may be observed. Again no noticeable difference was seen between cuvettes containing sulphite oxidase and those containing no sulphite oxidase. The only reaction observed was that between cytochrome c and sulphite oxidase.[29]

**Effect of sulphite Concentration on Cytochrome c Reduction**

As it was noted that an increase in cytochrome c reduction was observed with the addition of sodium sulphite to samples with and without the sulphite oxidase, a study on the effect on concentration was done. As the reaction is dependent on only the concentration of sodium sulphite, it is a first order reaction as shown by Perez-Benito et al.[29] and reported a kinetics study on the reduction of cytochrome c via sulphite in the neutral pH region (between pH 6.5 – 8.2). This reaction is written as:

$$2\ \text{cyt-Fe}^{3+} + SO_3^{2-} + H_2O \rightarrow 2\ \text{cyt-Fe}^{2+} + SO_4^{2-} + 2H^+$$

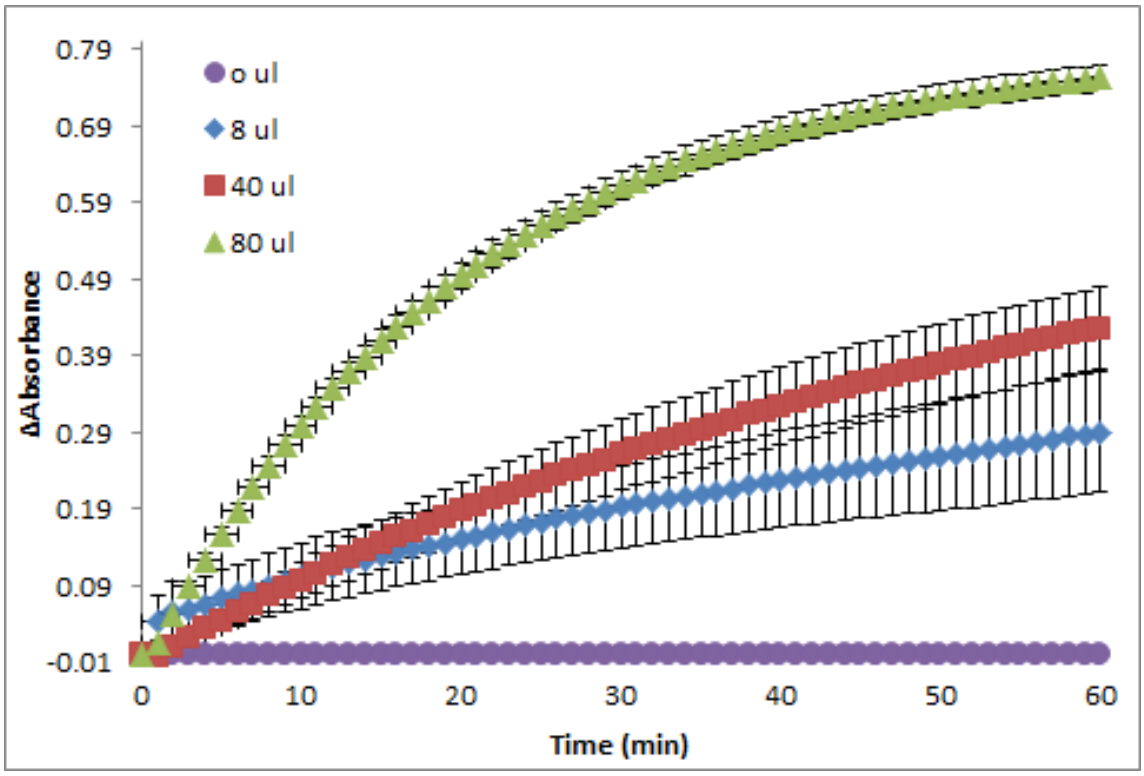



**Figure 29: The effect of increased Sulphite concentration on the reduction of cytochrome c by measuring the absorbance increase at 550 nm.[21]**

As expected, Figure 29 shows the increasing concentration increases the rate of cytochrome c reduction. This provides further evidence that the cytochrome c implemented into the biosensor does result in signal when exposed to sodium sulphite solutions and can possibly be used to detect sulphur in wines. This would provide a cheap and easy biosensor but the complex wine solutions have hindered further development. For this reason, work is continued on modifying the biosensor so it is active in wine solutions.

**Indirect Spectrophotometric Assay to detect the reduction of ferricyanide**

To determine if the molybdenum centre was active in the SUOX purchased from OriGene, the reduction of Potassium ferricyanide was observed by the decrease in absorbance at 420 nm.[21,25] Fresh stock solutions of 0.1 M of potassium ferricyanide and 0.1 M sodium sulphite in buffer solution were prepared. See table 3 for final solutions that were made.

### Table 3: Solutions made in cuvettes to measure absorbance

| Sample | | Solution | Solution | Amount (µL) | Solution | Amount (µL) | Solution | Amount (µL) | Total (µL) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | a | Buffer pH 4 | | | | | | | 2000 |
| | b | Buffer pH 4 | | | | | | | 2000 |
| | c | Buffer pH 4 | | | | | | | 2000 |
| 2 | a | Buffer pH 4 | 0.4 mM $Fe(CN)_6$ | 8 | | | | | 2000 |
| | b | Buffer pH 4 | 0.4 mM $Fe(CN)_6$ | 8 | | | | | 2000 |
| | c | Buffer pH 4 | 0.4 mM $Fe(CN)_6$ | 8 | | | | | 2000 |
| 3 | a | Buffer pH 4 | 0.4 mM $Fe(CN)_6$ | 8 | 0.1M $SO_3^{-2}$ | 8 | | | 2000 |
| | b | Buffer pH 4 | 0.4 mM $Fe(CN)_6$ | 8 | 0.1M $SO_3^{-2}$ | 8 | | | 2000 |
| | c | Buffer pH 4 | 0.4 mM $Fe(CN)_6$ | 8 | 0.1M $SO_3^{-2}$ | 8 | | | 2000 |
| 4 | a | Buffer pH 8 | 0.4 mM $Fe(CN)_6$ | 8 | 0.1M $SO_3^{-2}$ | 8 | SUOX | 90 | 2000 |
| | b | Buffer pH 8 | 0.4 mM $Fe(CN)_6$ | 8 | 0.1M $SO_3^{-2}$ | 8 | SUOX | 90 | 2000 |
| | c | Buffer pH 8 | 0.4 mM $Fe(CN)_6$ | 8 | 0.1M $SO_3^{-2}$ | 8 | SUOX | 90 | 2000 |

The absorbance at 420 nm was measured every 5 m until the all the solutions were stable (see Table 4). All the solutions remained stable over time except for samples containing sulphite oxidase, in which a linear decrease in absorbance was observed. After 15 min the potassium ferricyanide had reduced completely, indicating that the molybdenum domain in some of the sulphite oxidase is active (also see Figure 30).

**Table 4: SUOX activity measured by the decrease in absorbance a tλ 420 nm relating to the reduction of potassium ferricyanide.**

| Samples | | Time (min) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 5 | 10 | 15 | 20 |
| Buffer | 1 | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 |
| | 2 | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 |
| | 3 | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 |
| | AV | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 |
| | Std | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Buffer + $Fe(CN)_6$ | 1 | 0.55 | 0.55 | 0.55 | 0.55 | 0.55 |
| | 2 | 0.58 | 0.58 | 0.58 | 0.58 | 0.58 |
| | 3 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 |
| | AV | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 |
| | Std | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Buffer + $Fe(CN)_6$ $+SO_3^{-2}$ | 1 | 0.55 | 0.55 | 0.55 | 0.55 | 0.55 |
| | 2 | 0.53 | 0.52 | 0.52 | 0.52 | 0.52 |
| | 3 | 0.55 | 0.55 | 0.55 | 0.55 | 0.55 |
| | AV | 0.54 | 0.54 | 0.54 | 0.54 | 0.54 |
| | Std | 0.01 | 0.01 | 0.02 | 0.01 | 0.02 |
| Buffer + $Fe(CN)_6$ $+SO_3^{-2}$ + SUOX | 1 | 0.56 | 0.51 | 0.28 | 0.16 | 0.16 |
| | 2 | 0.56 | 0.40 | 0.23 | 0.17 | 0.17 |
| | 3 | 0.55 | 0.36 | 0.19 | 0.16 | 0.16 |
| | AV | 0.56 | 0.43 | 0.24 | 0.16 | 0.16 |
| | Std | 0.01 | 0.08 | 0.05 | 0.00 | 0.00 |

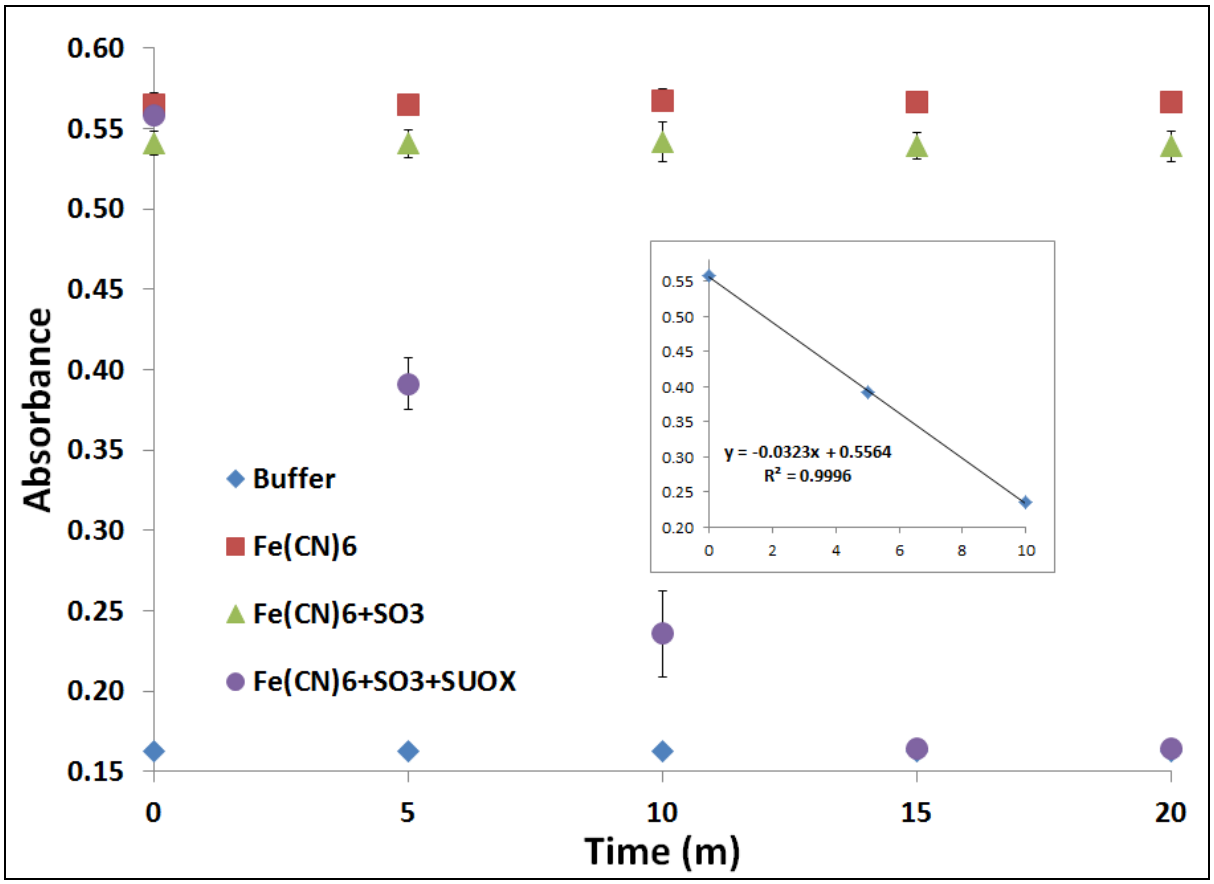



**Figure 30: Graph showing the activity of sulphite oxidase by measuring the absorbance reduction at 420 nm due to the reduction of potassium ferricyanide.**

As one unit of sulphite: ferricyanide activity is defined as the amount of enzyme required to yield an absorbance change of 1.0/min at 25 $^{o}$C, the calculated activity of sulphite oxidase can be determined by the slope. The activity of 6.57 µg of enzyme was 0.03 /min (see Figure 30 insert), thus the specific activity was 4.6 units/mg. This is quite low compared to other purified enzymes (see Table 1) but could be sufficient for a biosensor as long as the enzyme is active once immobilised on the electrode.

### Summary of Attempt to Produce Active, Purified hSUOX

The commercially available human sulphite oxidase (SUOX) used in our laboratory was inactive; therefore the enzyme was overexpressed in house to attempt to isolate active enzyme. A molybdenum cofactor, required for optimal enzyme activity, is incorporated into one domain of SUOX. To ensure high yields of protein expression, enzyme activity, and molybdenum cofactor incorporation, it was planned to overexpress SUOX in *Escherichia coli* TP1000. The TP1000 strain has deletions in *mobA* and *mobB* genes, permitting greater molybdenum cofactor incorporation into overexpressed SUOX-myc-flag than would be obtained in wild type *E. coli* strains. Arrangements were made to ship *E. coli* TP1000 to Flinders University from the Palmer Laboratory in the University of Dundee, Scotland. While obtaining the necessary permits and material transfer agreements required to import the TP1000 strain into Australia, SUOX was transfected into the human cell lines, HEK293T and HEK293A.

HEK293T cells were transfected with the human SUOX gene, fused to both myc and flag tags (pCMV6-SUOX-myc-flag; Origene, USA); however, no SUOX was detected by Western blot in these transfected cells. This was repeated in HEK293A cells following established protocols; however the cells were sensitive to the transfection conditions and died as a result of the transfection reagent. Repeated studies using less transfection reagent resulted in large amounts of cell death, and no detectable SUOX expression.

As the transfection toxicity was unexpected, and the pCMV6 vector had not previously been used, SUOX-myc-flag was subcloned into the pIRES vector, a eukaryotic expression vector known to be tolerated in the HEK293 cells used in this lab. Site directed mutagenesis was used to introduce a new restriction enzyme recognition site, XbaI, required for subcloning SUOX-myc-flag out of the original pCMV6 vector. Once validation steps were taken to ensure the XbaI site was introduced into the pCMV6-SUOX-myc-flag, it was sent for DNA sequencing for confirmation. SUOX-myc-flag was subsequently subcloned into the pIRES vector, confirmed by restriction digests and DNA sequencing, generating pIRES-SUOX-myc-flag. HEK293T cells were transfected with pIRES-SUOX-myc-flag, but no SUOX was detected by Western blot. A final experiment determined that cells were not confluent enough at time of transfection in previous experiments.

Site directed mutagenesis was used to introduce suitable restriction sites into pIRES-SUOX-myc-flag to facilitate subcloning into the bacterial expression vector, pCWOri+, validated by restriction digests and DNA sequencing. pCWOri+-SUOX-myc-flag was transformed into *E. coli* TP1000, and SUOX-myc-flag protein was overexpressed in *E. coli* TP1000, as identified using Western blot. Bacterial culture conditions were optimised to increase yield of total protein, while cell lysis and protein isolation steps were also optimised to maximise total protein recovery and SUOX activity. Low levels of SUOX activity were detected in all cell lysates. Isolation of SUOX-myc-flag was attempted using a flag-tag purification kit designed to specifically isolate flag-containing protein; however, no SUOX activity was detected in the isolated sample or the total cell lysate. Further studies indicated that initially observed SUOX activity was not due to the enzyme, as the activity did not change with protein concentration.

## 7. Outcome/Conclusion:

At the commencement of this project, it was important that the previous biosensor developed could be replicated and the results obtained previously were reproducible. Initially the biosensors were reproduced and tested so further modification could be done to overcome the inhibition of the signal caused by the addition of wine. This testing addressed the first milestones of the project.

Testing the sensor in wine was the next milestone and not successful. A great deal of work explored the important issues for the failure and there is still work to be done in this area. It was found that the purchased enzyme was not the correct protein and so the signal response was caused by a different component (for example, cytochrome c). It was thought that modifying the biosensor without the sulphite oxidase could be achieved and another source of sulphite oxidase could be investigated. Modifications such as implementing gold nanoparticles and polymer membrane could be used to create a more stable biosensor that could be used in complex wine solutions. Further investigation found that although sulphite and cytochrome c did react (sulphite oxidised to sulphate and cytochrome c reduced), the signal received may be background noise caused by the gold and sulphite interacting. With that said, it is hoped that the methods that were trialled could be implemented in future work to create a more stable and specific biosensor.

This research addressed measurement of $SO_2$, one of the most widespread and fundamental wine quality measurements and as such has high significance to producers and consumers across many levels of the wine community. Sulphur dioxide is added to wine at nearly every stage of production and serves as both an anti-microbial agent and an antioxidant. When used effectively it is also one of the simplest ways to minimise quality loss. Currently, sulphur dioxide measurement is time consuming and costly (particularly in terms of staff time or outsourcing) and cheaper, rapid, portable methods are desirable.

This final objective of this project was to deliver a biosensor that can readily and reliably measure sulphite concentrations in wine at every step of the production process. We have made progress in this area. The sensor was based on an electronic signal from an electrochemical reaction and would likely require removal of a small sample to be added to a provided solution which is then tested. This would drastically lower the effort and financial burden of sulphite testing and would be of great benefit to the wine industry.

## 8. Recommendations:

The use of the sensor in wine samples was unsuccessful with no signals observed. This was thought to be due to the fouling from something in the complex wine matrix. Several sources of interferents were explored including ethanol and polyphenols. The sensors still did not work without these species present so further work is required to explore the nature of the deactivation of the sensor. There can be little doubt of the importance of sulphite sensing in the wine industry. As such continued investment in the development of such a sensor is well worth the investment and the follow section details some possible avenues for exploration.

Once these inferences are understood there is ready scope to build a new sensor that will work in the wine matrix. Some possible avenues forward include the use of a different enzyme or detection system, addition of an antifouling layer, controlling of the morphology of the sensor to reduce certain adsorption, building an architecture where the environment of the sensing element can be controlled and maintained more readily etc.

This work has great potential and presents the exciting possibility of developing a cheap, easy to use and reusable sensor for sulphite in wine and in fact other beverages. The successful development of such a device would have a global impact on the wine industry.

Overall, the initial biosensor that was developed had many flaws but through the work done since Feb 2012, a good foundation has been developed to get the biosensor functional. For example, it may be possible to create a biosensor with using just cytochrome c or just sulphite oxidase (in the correct orientation) that maintained protein functionality.[5,26]

The effect of increased sulphite concentration on the cyclic voltammogram scan of bare gold was scanned (Figure 32) and an increased signal was observed similar (but not transient) to that observed previously. This needs to be further investigated by comparing 1) bare gold, 2) bare gold with cysteamine SAM and 3) bare gold, cysteamine with immobilised cytochrome c to fully determine the cause of the signal response observed with sulphite additions. It is thought that the Au was somehow interacting with the sulphite that this would not be seen in the presence of the SAM layer, as there should be close to 100% coverage but possibly the SAM layer delaminates exposing the bare gold.

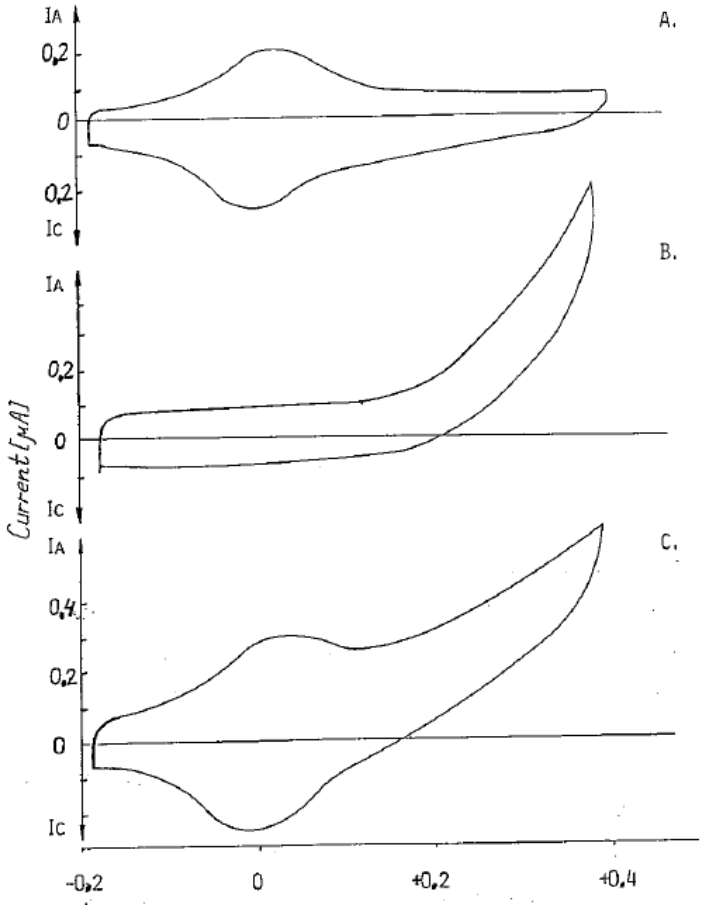



**Figure 31: Cyclic voltammogram for solutions containing A) cytochrome c, B) Sodium Sulphite and C) both cytochrome c and sodium sulphite. Reference 26.**

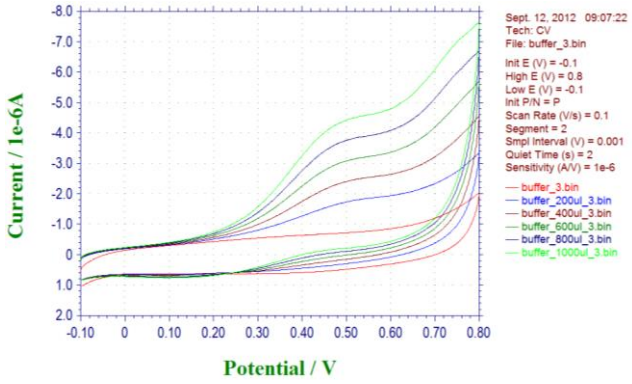



**Figure 32: Cyclic voltammogram of bare gold surface with additions of sodium sulphite.**

### Cytochrome c Orientation and Functionality

When attaching a protein to an electrode surface it is important to ensure that the protein does not become denatured and the orientation allows the protein to remain functional.[30,31] For this reason, functionalised spacers such as self-assembled monolayers with varying head groups can be formed on the electrode surface to conserve the native state and promote order of the specific protein.[32] For the naturally basic protein Cytochrome c, many studies have been done

to determine the optimum surface chemistry that would maintain the native state of the protein whilst still allowing the heme group to be accessible. Work done by Haas *et al*. established that an alcohol rich surface ordered the cytochrome c with the heme group orientated on the surface, where a carboxylic acid functionalised surface orientated the heme group down (towards the surface of the electrode). More importantly, a functionalised surface of thiol groups caused the proteins to become denatured once immobilised on the surface. This was further employed by Spricigo et al.[5], who used a combination of alcohol and carboxylic acid groups on the surface to create an optimum surface for signal response with sulphite oxidase.

For future modifications to the current biosensor, the positioning and orientation of the cytochrome c needs to be considered, thus the effect of the functionalised self-assembled monolayers used to immobilise the protein should be fully investigated by trialling varying the chemistry of the head group and combining different functionalities to maintain the natural state of cytochrome c and subsequent sulphite oxidase enzymatic response (discussed later). The heads group's trialled should be negatively charged (as cytochrome c is naturally basic) but incorporating neutral components for steric hindrance (as one example) could also be tested.

**Incorporating Sulphite Oxidase**

The sulphite Oxidase purchased from OriGene did not show any activity with the cytochrome c but did however show activity with the reduction of ferricyanide**.** A more direct method should be implemented to test its ability to oxidise sulphite to sulphate. This should be done as in Table 2 in which this reaction can be seen by the reduced absorbance at 212nm. Work undertaken by Flinders to purify the recombinant enzyme would potentially provide higher yields with higher activity once successful.

When incorporating sulphite oxidase, many variables need to be considered such as the placement (in relation to electron acceptor and the electrode surface) and the orientation (ensuring the active section of the protein is accessible). Not much work has been done on this specifically as not all biosensors containing sulphite oxidase involves the use of cytochrome c as an electron acceptor. Previous work done which incorporates cytochrome c has established the importance of the ratio of cytochrome c and sulphite oxidase and having the sulphite oxidase on the surface of the sensor.

**Poly (ethylene glycol) bio fouling film**

The suflite oxidase and/or cytochrome c could be immobilised onto the surface of the electrodes using polyethylene glycol (PEG), creating an anti-fouling membrane/film. PEG is a well-known polymer that prevents the absorption of proteins that may cause the fouling of membranes. Incorporating cytochrome c into the film may provide a biosensor that will have a signal in wine solutions. This will be trialled with the current biosensor, which involves the self-assembled monolayer (SAM) of cysteine[33] and absorption of 19 nm gold nanoparticles (followed by cytochrome c attachment). This biosensor may be altered to further improve the signal and stability.

**Appendix 1: Communication:**

The only communication at this stage has been GWRCD reports.

**Appendix 2: Intellectual Property:**

Identify the intellectual property and/or valuable information arising from the research.

The background knowledge of the Flinders honours project has not be subject to IP protection and has been the subject of public presentations and hence no longer protectable. Our background knowledge from Flinders was the subject of publications some years ago. In both cases, the approach to build enzymatic electrochemical sensors is described and the successful detection of glucose (see reference following) and sulphite (2010 Honours Thesis) is demonstrated.

D. Losic, J.J. Gooding, J.G. Shapter, D.B. Hibbert and K. Short "The Influence of the Underlying Gold Substrate on Glucose Oxidase Electrodes Fabricated using Self-assembled Monolayers" Electroanalysis **13** 1385-1393 (2001).

The current work has not generated any IP but the groundwork has been laid, problems identified and a clear path forward suggested to develop a sensor that would be critical the wine industry and such the IP would be of great interest and value. The sensor would be cheap to manufacture, easy to use in the field and provide accurate measurement of sulphite level. These developments would without a doubt be patentable.

**Appendix 3: References**


(1) Rankine, B. C.; Pocock, K. F. Australian Wine, Brewing and spirit review 1970, 88, 40.
(2) Cardwell, T.; Nan, C. G.; Scollary, G.; Sneyd, N. The Australian Grapegrower and Winemaker 1989.
(3) Sneyd, T. N.; Leske, P. A.; Dunsford, P. A. Proceedings of the Eighth Australian Wine Industry Tehcnical Conference; 25-29 October 1992, 161.
(4) Bahmani, B.; Moztarzedeh, F.; Rabiee, M.; Tahriri, M. Synthetic Metals 2010, 160, 2653.
(5) Spricigo, R.; Dronov, R.; Lisdat, F.; Leimkühler, S.; Scheller, F.; Wollenberger, U. Analytical and Bioanalytical Chemistry 2009, 393, 225.
(6) Ameer, Q.; Adeloju, S. B. Electroanalysis 2008, 20, 2549.
(7) Abass, A. K.; Hart, J. P.; Cowell, D. Sensors and Actuators B 2000, 62, 148.
(8) Situmorang, M.; Brynn Hibbert, D.; Justin Gooding, J.; Barnett, D. Analyst 1999, 124, 1775.
(9) Dronov, R.; Kurht, D. G.; H., M.; Spricigo, R.; Leimkuhler, S.; Wollenberger, U.; Rajagopalan, K. V.; Scheller, F.; Lisdat, F. Journal of the American Chemistry Society 2008, 130, 1122.
(10) Rawal, R.; Chawla, S.; Dahiya, T.; Pundir, C. Analytical and Bioanalytical Chemistry 2011, 401, 2599.
(11) Kalimuthu, P.; Tkac, J.; Kappler, U.; Davis, J. J.; Bernhardt, P. V. Analytical Chemistry 2010, 82, 7374.
(12) Dinckaya, E.; Sezginturk, M. K.; Akyilmaz, E.; Ertas, F. N. Food Chemistry 2007, 101, 1540.
(13) Losic D. et al. "The Influence of the Underlying Gold Substrate on Glucose Oxidase Electrodes Fabricated using Self-assembled Monolayers" Electroanalysis **13** 1385-1393 (2001).
(14) Xiang, C.; Zou, Y.; Sun, L.-X.; Xu, F. Electrochemistry Communications 2008, 10, 38.
(15) Turkevich, J.; Stevenson, P. C.; Hillier, J. Discussions of the Faraday Society 1951, 11, 55.
(16) Zhu, T.; Vasilev, K.; Kreiter, M.; Mittler, S.; Knoll, W. Langmuir 2003, 19, 9518.
(17) Goreham, R. V.; Short, R. D.; Vasilev, K. Journal of Physical Chemistry C 2011, 115, 3429.
(18) Losic, D.; Shapter, J. G.; Gooding, J. J. Journal of Solid State Electrochem 2005, 9, 512.
(19) Bilen, S.; Dick, W. A. Biol. Fertil. Soils 2011, 47, 647.
(20) Buck, R. P.; Singhadeja, S.; Rogers, L. B. Analytical Chemistry 1954, 26, 1240.
(21) Cohen, H. J.; Fridovich, I. The Journal of Biological Chemistry 246, 246, 359.
(22) MacLeod, R. M.; Farkas, W.; Fridovich, I.; Handler, P. The Journal of Biological Chemistry 1961, 236, 1841.
(23) Garrett, R. M.; Rajagopalan, K. V. The Journal of Biological Chemistry 1996, 271, 7387.
(24) Johnson, J. L.; Rajagopalan, K. V. Journal of Clinical Investigation 1976, 58, 543.
(25) Temple, C. A.; Graf, T. N.; Rajagopalan, K. V. Archives of Biochemistry and Biosphysics 2000, 383, 281.
(26) Benov, V. P.; Atanasov, B. P. Analytical Letters 1993, 26, 2061.
(27) Bernhardt, P. V. Australian Journal of Chemistry 2006, 59, 233.
(28) Liu, Y.; Wu, S.; Ju, H.; Xu, L. Electroanalysis 2007, 19, 986.
(29) Perez-Benito, J. F.; Arias, C. Collect. Czech. Chem. Commun. 1990, 56, 1552.

(30) Sakurai, T.; Fumitaka, N. Chemistry Letters 1995, 12, 1075.
(31) Anirudhan, T. S.; Senan, P. Chemical Engineering Journal 2011, 168, 678.
(32) Haas, A. S.; Pilloud, D. L.; Reddy, K. S.; Babcock, G. T.; Moser, C. C.; Blasie, J. K.; Dutton, P. L. Journal of Physical Chemistr B 2001, 105, 11351.
(33) Gooding, J. J.; Wibowo, R.; Lui, J.; Yang, W.; Losic, D.; Orbons, S.; Mearns, F. J.; Shapter, J. G.; Hibbert, D. B. JACS communications 2003, 125, 9006.

**Appendix 4: Staff** List all staff that have been engaged on the Project.

Research work undertaken by Jared Parsons, Cameron Shearer, Vanessa Thompson, Renee Goreham and Jayant Patel.

**Appendix 5:** Any other relevant material including eg raw data.

Available electronically if required.

**Budget reconciliation** The End of Project Financial