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Effects Of Ozone On Blood Components

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by

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Dedication

To my husband and parents for all their love and support

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Acronyms

CC16 protein – Clara cell serum protein

CHD – coronary heart disease

CRP – C-reactive protein

hsCRP – high sensitivity C-reactive protein

DTNB - 5,5'-Dithio-Bis 2-nitrobenzoic acid

ESR – erythrocyte sedimentation rate

GSH –glutathione (reduced)

GSHPx – glutathione peroxidase

GSSH – glutathione (oxidized)

IgG - Immunoglobulin

MPA – metaphosphoric acid

NADPH - nicotinamide adenine dinucleotide phosphate

NOS2 – nitric oxide synthase

PAHs – polyaromatic hydrocarbons

SOD – superoxide dismutase

TMB - 3,3’, 5,5’-tetramethylbenzidine

VOCs – volatile organic compounds

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ABSTRACT

Previous studies on the medical use of ozone therapies show a very diverse array of results, from ozone reducing the amount of HIV virus in the blood, to no effect, to causing the death of several patients due to pulmonary embolism and infections. However, ozone therapies are widely used in Europe and considered medically safe. In the U.S., doctors in 28 states use ozone therapies.

The objectives of this study were to investigate the effects of medical grade ozone at varying concentrations used in ozone therapies. These were achieved by evaluating the C-reactive protein, erythrocyte sedimentation rate, total reduced and oxidized glutathione content of erythrocytes which were all markers used to determine ozone injury/inflammation.

Despite the fact that ozone is a very strong oxidant, previous research indicates that depending on the dose and the health status of the biological system, sometimes ozone can act as an antioxidant.

The medical exposure range for ozone is between 20 -80 μg/ml with an average of 50 μg/ml. The concentrations used in this study were 20, 40, 80 and 160 μg/ml.

Ozone was generated in the “Breath Lab” at USF from medical grade oxygen obtained through electrical corona arc discharge using an OL80C ozone generator. De-identified blood samples of 10 ml blood/sample containing EDTA as anticoagulant were obtained from the James A. Haley VA Hospital patients. Equal volumes of blood and ozone gas mixture were allowed to mix in ozone-resistant syringes prior to dividing each sample into three parts, one for each corresponding parameter to be studied. The C-reactive protein was analyzed through ELISA using the colorimetric method available from Helica Biosystems; erythrocyte sedimentation rate was measured in graduated sedimentation tubes; the total reduced glutathione (GSH) and oxidized glutathione (GSSG) content of erythrocytes was determined according to the colorimetric method developed by the Oxford Biomedical Research.

Overall, the concentrations of ozone used did not have a statistically significant effect on the parameters investigated. However, a small percentage of the blood samples showed an improvement in the parameters studied, especially at the highest ozone concentration.

Introduction

Background

Ozone is one of the five major air pollutants along with carbon monoxide, sulfur dioxide, nitrogen dioxide and particulates. The difference between ozone and these four pollutants is the fact that it is not emitted directly into the air from industrial facilities, power plants or automobiles. Instead, ozone is a photochemical pollutant, a major component of photochemical smog, created when the sunlight mediates chemical reactions with other pollutants (Breslin, 1995). Due to this effect, human exposures to atmospheric ozone are of interest and thus have been studied for a long time. Most commonly the effects of ozone are lung injury, respiratory infections and inflammation caused by ozone concentrations of 0.1 ppm in adults and as little as 0.085 ppm in children. These findings prompted the USEPA to revise its 0.12 ppm standard and set a limit of 0.08 ppm ozone/8 hours, based on the decision that this level protects the public health (Moore 1999). However, studies on southern Californians showed that the pulmonary function changes at 0.5 ppm are less severe during a high-ozone season compared to a low-ozone season, suggesting an increased tolerance to ozone but not less cellular damage (Munzer et al., 1995). On the other hand, ozone has been widely used in the medical field for ozone therapies, the most common being ozone autohemotherapy or ozonated autohemoadministration (Hiromichi et al. 2006,

en.wikipedia.org/wiki/Ozone_therapy). Autohemotherapy is an alternative medical technique practiced for about 50 years in Europe, which involves withdrawing up to 200 ml of venous blood, then immediately mixing it with therapeutic concentrations of ozone gas and a minimal amount of anticoagulant, usually heparin, then re-injecting it into the basilic vein at the elbow. Other techniques are ozone bagging, when all parts of the body except for the head are placed in a bag full of ozone at a concentration of 100 μg of ozone /ml air mixture for up to 2 hours; ozone rectal insufflation, where an average of about 1 1/2 liters of 27 μg/ml O_3 gas are introduced into the colon; ozone vaginal insufflation, where the vagina is insufflated for about 5 minutes; ozone ear insufflation, where O_3 is introduced in the ear cavity for an average of 5 minutes; ozone air purification, where low levels of ozone sterilize and rejuvenate the room air and lastly ozone charged drinking water, where O_3 is bubbled into water which must be imbibed immediately while the O_3 is still in the glass (oxygenmedicine.com). Experimental evidence suggests that these therapies may boost the immune system, reduce the number of viruses in the blood and a reduction in lung, breast and uterine tumors (Sweet et al. 1980). A study by Wells et al. (1991) demonstrated that ozone was able to inactivate HIV-1 virions in a dose- dependent manner. Ozone concentrations of 1200 ppm achieved greater than 11-log virus inactivation within 2 hours from ozone administration (2-log means 99% inactivation). The authors developed and used a T cell line – HUT 78/HIV-1_{AAV} stably infected with HIV-1. The ozone was delivered into the cell medium through a closed hollow fiber system as a stream of ozone/oxygen, using nitrogen as the carrier.

Ozone therapy is used legally in 16 countries, mostly in Europe. In the U.S., recently passed Alternative Therapy Legislation has made ozone therapy an option for patients in

13 states. In Alaska, Arizona, Colorado, Georgia, Minnesota, New York, New Jersey, North Carolina, Ohio, Oklahoma, Oregon, South Carolina, and Washington, physicians can legally use ozone treatments in their practice without fear of prosecution. (wikipedia.org).

Scientific papers that described studies on the effects of ozone and/or ozone therapies in relation to blood provided contradicting results. First, there are the studies that found no effect of ozone on blood and blood constituents. Biedunkiewicz et al. (2006) found no evidence that ozone affects blood coagulation and fibrinolysis. It would have been expected that ozone reduces blood viscosity and inhibits coagulation, which are important side effects for patients undergoing hemodialysis. Autohemoadministration lead to no statistical differences between C-reactive protein at baseline and after ozone treatment in patients undergoing hemodialysis (Tylicki et al. 2004). This result proved that autohemoadministration is safe for the patients. Zimran et al. (1999) showed that ozone does not affect red blood cell enzymes and intermediates or red blood cell integrity. Furthermore, ozone neither damage erythrocytes, nor induced oxidation of intracellular hemoglobin in the case of heparin-treated blood (wikipedia.org). A study by Travagli et al. (2006) did not yield significant hemolysis or methemoglobin when whole blood was treated with a therapeutic concentration of ozone. In contrast, there are studies that showed either a negative or a positive effect of ozone. Bocci et al. (1999) showed that during ozonated autohemotherapy, ozone induced formation of platelet aggregation (blood clots) in heparin (anticoagulant)-treated blood (Bocci et al. 1996). Larini and Bocci (2004) showed that cytokine production was depressed at ozone concentrations above 40 μg/ml. Bocci (2006) advocated the use of ozone therapies and ending the

labeling of ozone therapies as a dangerous or toxic, while recognizing that atmospheric ozone can be responsible for respiratory system damage. In this case, ozone therapies were shown to improve blood circulation and oxygen delivery to ischemic tissues and induce a mild activation of the immune system (Bocci 2006). The same author, Bocci (2007) later demonstrated that ozone can activate biochemical pathways in leukocytes, erythrocytes and platelets without acute or chronic toxicity, and decreases blood plasma antioxidant capacity for about 20 minutes (Bocci, 2007). Patients under maintenance hemodialysis who were given autohemoadministration showed a decrease in blood access recirculation, which is the return of the dialyzed blood into the extracorporeal circuit through the arterial needle, rather than returning to the systemic circulation. This is a positive effect, helping to maintain the effectiveness of hemodialysis, even though these results were not statistically significant (Tylicki et al. 2004).

Blomberg et al. (2003) showed that exposure to atmospheric ozone impairs lung function, induces airway inflammation and alters epithelial permeability, as shown by analysis of CC16 protein from peripheral blood (Blomberg et al. 2003). Animal studies showed that ozone exposure results in local bronchial inflammation and also affects the nervous system and thymocyte proliferation, and places mice under oxidative stress (Feng et al. 2006). In rats, ozone exposed animals had an increased lyzozyme activity and a decreased total protein, both being an indicator of liver disease (Jakubowski et al. 2004). Another study on mice by Kenyon et al. (2006) demonstrated that ozone induced acute lung injury but the NOS2 enzyme present in some mice had a protective effect against lung injury. Experiments on male rats resulted in a positive linear relationship between ozone concentration and the concentrations of serum total lipoprotein free

cholesterol (FCh) and high-density lipoprotein total cholesterol (HDL-Ch) (Mole et al. 1985). A study reported by Hiromochi et al. (2006) on the effects of ozone autohemoadministration in cows, showed significant changes in leukocyte populations following ozone blood stimulation (Hiromichi et al. 2006). Other studies showed were that exposure to ozone increases sensitivity to the toxicity of other chemicals like 1-nitronaphtalene (Schmelzer et al. 2006); external ozone exposure combined with internal exposure to PAHs and VOCs resulted in low level of DNA damage in teenagers but it is not clear if ozone alone can be responsible for the mutations (Koppen et al. 2007). Air pollutants, including ozone, were shown to cause pulmonary inflammation in both human and animals under experimental conditions; this causes an increase in the liver inflammatory markers, fibrinogen and C-reactive protein. However, when air pollutant exposure of 40 healthy volunteers was studied over the course of a 1-year period, there was no relationship between air pollutants and the amount of fibrinogen and CRP (Rudez et al. 2009). Lab experiments on healthy volunteers showed that exposure to an ambient air ozone concentration of 0.5 ppm induced a significant decrease in vital capacity and total lung capacity, expiratory flow rates and an increase of respiratory frequency on exercise (Hazucha et al. 1989).This concurs with a study by Bowler and Crapo (2002) showing that ozone exposure decreases the forced expiratory volume FEV_1 and children playing in areas with high concentrations of environmental ozone have a higher incidence of asthma. Despite these results, a review of 24 studies concluded that a threshold concentration below which no effects on pulmonary function are elicited cannot be defined (Hazucha, 1987).

A positive effect of ambient ozone was noticed when cancer cells extracted from lung, breast and uterine tumors were exposed to ozone concentrations between 0.3 to 0.8 ppm in ambient air. Concentrations between 0.3 to 0.5 ppm inhibited cancer growth between 40 and 60 percent. A concentration of 0.8 ppm inhibited cancer cell growth more than 90 percent. This shows that human cancer cells have an impaired defense mechanism against ozone, compared to normal cells (Sweet et al. 1980).

A summary of some of the previous studies on ozone therapies and their effects, positive, negative or no effect is given below in Table 1.

Table 1. Summary of Previous Studies on Ozone.

Previous studies	Sample size	Positive effects	Negative effects	No effect
Guven et al. (2009)	rats	Reduced intestinal damage, oxidative stress		
Rodriguez et al. (2009)	rats	Increase in antioxidant enzymes, decrease myeloperoxidase (damage marker) in lungs		
Labuschagne et al (2009)	baboons	Up-regulated antioxidant capacity		
Schultz et al. (2008)	rabbits	Remission of squamous cell carcinoma		
Jiao & Peng (2008)	42	Improvement in liver function for hep. B		
Mustafaev et al. (2007)	20	Prevention of pyoinflammatory complications following transurethral resection of prostatic adenoma		
Ohtsuka et al. (2006)	cows	Increased $CD4^+/CD8^+$ ratio		
Jakubowski et al. (2004)	rats	Increased lysozyme activity, decreased total protein level		
Clavo et al. (2004)	18	Improved oxygenation in hypoxic tumors		
Simonetti et al. (2003)	600	Additive effect for lumbar disk herniation		
Al-Dalain et al (2001)	rats	Improvement in glycemic control		
Sweet et al. (1980)	cells	Inhibited cancer cell growth		

Table 1 (Continued)

Blomberg et al. (2003)	22		Increased CC16 serum (marker for ozone-induced lung damage)	
Hazucha et al. (1989)	14		Inhibited inspiration, reduced total lung capacity and vital capacity	
Forsberg et al.	3430		Increased blood fibrinogen	
Gornicki & Gutse (2000)	cells		Lead to changes in erythrocyte membranes, cytoskeletal proteins	
Schmelzer et al. (2006)	rats			On cytokine production
Biedunkiewicz et al. (2006)	11			On blood coagulation parameters
Tylicki et al. (2004)	12			On inflammation response in hemodialyzed patients

The main difference between the studies that did not find any effects of ozone on blood and the studies that found a wide diversity of effects, can be attributed to the different methodology used. In general, the findings of the studies with no effects were based on a single concentration of ozone to work with; whereas the studies that were able to prove that ozone had either a positive or negative effect looked at several ozone concentrations. However, the latter analyzed a specific problem such as heparinized blood from male donors (wikipedia.org), heparinized or citric acid treated blood from volunteers between 23 and 27 years old, plasma (Bocci et al. 1999), release of cytokines from mononuclear cells (Larini & Bocci 2005), total cholesterol in male rats (Mole et al. 1985) or methemoglobin (Bocci & Aldinucci 2006). Another possible explanation for the in vitro damaging effect of ozone on blood components is that the ozone toxicity is exerted when cells are incubated in anti- oxidant-free culture media and therefore do not benefit of the antioxidant capacity of the blood (Larini & Bocci, 2005).

According to Hernandez (2007), one of the reasons why ozone in medicine has not been approved as a common practice is its use without an appropriate control. The main ozone therapy mechanism of action is based on an extremely transitory and regulated oxidative stress imposed ex vivo (Bocci, 2002). At the same time, ozone therapy acts as an efficient oxidative stress regulator stimulating the antioxidant system of the cell. As reactive oxygen species attack a variety of organic substrates, oxidative stress can be evaluated by measuring reaction products of oxidative damage. Because of this, it would be necessary to assess the patient's redox status before and during application of ozone therapy in order to control the safety doses of ozone to be applied in each application.

Previous reports remarked on the lack of studies on the effects of ozone on the

immune responses, on peripheral blood leukocytes, the mechanism of ozone therapies and that there are controversies on whether the blood should be diluted or not. Furthermore, while the fact that ozone damages the membrane of erythrocytes is well known, the ozone therapies are considered by many as safe but others claim that ozone concentrations within the medical range cause degradation of the proteins in erythrocyte membranes (Fischbach, 2000).

Common markers of inflammatory responses to infections and chemical agents are an increase in C- reactive protein and a short-term increase in GSH (glutathione) levels. Glutathione is an antioxidant that protects cells from toxins such as free radicals generated by the powerful oxidative properties of ozone. The C-reactive protein is usually absent in the blood of healthy persons and appears rapidly in blood and body fluids as a response to injurious stimuli (Fischbach, 2000). However, another study by Ridker et al. (2000) showed that high levels of the high sensitivity C-reactive protein were found in the blood of healthy postmenopausal women. Later on, these women developed various forms of cardiovascular disease and hsCRP was the significant predictor of cardiovascular risk out of 12 plasma variables. This result confirmed a previous study (Kuller et al., 1996) which was the first to show a direct correlation between CRP and coronary heart disease (CHD) mortality in healthy but high risk men. The correlation between CRP and CHD mortality is strengthened when other risk factors like smoking are present. However, the results of this study were not able to show a correlation between CRP and nonfatal myocardial infarctions, only a correlation between CRP and CHD deaths. Ridker et al. (2005) pursued this topic and for high sensitivity assays of CRP, their cut-offs were less than 1 mg/L for low risk, 1 to 3 mg/L for

moderate risk, and greater than 3 mg/L for high risk. The drawback is that the continuum extends beyond that. The patients with the very highest levels of hsCRP —5 to 10, 10 to 20, or even greater than 20 mg/L—are at the very highest risk. These were not false positives and they helped to explain why people with periodontal disease, arthritis, and other systemic inflammatory disorders had higher vascular risk. A plausible explanation is that inflammation from any cause has an adverse effect on the vascular endothelium.

Antioxidant enzymes like copper/zinc superoxide dismutase (SOD), catalase and gluthatione peroxidase (GSHPx) are part of the intracellular protection mechanism important in overcoming oxidative stress and are known to be activated in vascular diseases and acute stroke (Zimmermann et al. 2004). In this study, nearly two thirds of the patients with a stroke in the past showed decreased GSH levels and the authors speculate it was possibly associated with increased oxidative stress and arteriosclerosis. The GSH levels and antioxidant capacity are also decreased following an organ transplant, which may indicate the need for glutathione supplementation to improve antioxidant status (Wierzbicka et al. 2007). Glutathione (GSH) is an important tripeptide thiol (γ-glutamyl cysteinyl glycine) antioxidant and its intracellular concentration is indicative of oxidative stress. The oxidative stress is a common marker of many diseases such as chronic lung diseases, neurodegenerative diseases rheumatoid arthritis, amyotrophic lateral sclerosis and most recently AIDS (Rahman et al. 2005, Halliwell 1996). Within the cell glutathione is found in two forms: GSH, the reduced sulfhydryl form and GSSG, the oxidized disulfide form (Rahman et al. 2005). A summary of some of the previous studies on ozone therapies and their effect on blood is given below in Table 2.

Table 2. Summary of Previous Studies on Blood and Ozone.

FINDINGS FROM PREVIOUS STUDIES	SAMPLE SIZE	EFFECTS OF OZONE ON			
		ESR	CRP	GSH	OTHER
Bocci et al. 2006	1	n/a	n/a	n/a	Total antioxidant status decreased temporarily
Travagli et al. 2007	3	No effect		Decrease in GSH enzymes but not significant	Decrease in total antioxidant status, no effect on fibrinogen, cholesterol
Bocci et al. 1999	5			No effect	Decrease in total antioxidant status, reversible platelet aggregation
Biedunkiewicz et al. 2006	11				No effect on blood coagulation, fibrinolysis
Tylicki et al. 2004	12		No effect		
Gornicki & Gutsz, 2000	21?				Effect on erythrocyte membrane fluidity is dose dependent
Clavo et al. 2004	18				Ozone increased tumor oxygenation
Goran et al. 2009	40		No effect		Ozone increased platelet aggregation, thrombin generation
Mustafaev et al. 2007	20	Decrease			Increase in leukocytes, phagocytes
Haddad et al. 2009	horses				Decrease in gamma glutamyltransferase, increase in fibrinogen
Ohtsuka et al.2005	cows				Increase in plasma protein, serum protein, α and γ globulin, CD4+T cells

Table 2 (Continued)

Lambuschagne et al. 2009	baboons			Decrease in total GSH	
Rodriguez et al. 2009 -O_2/O_3 mix insufflated in to lower abdomen, not blood	rats			Increase in GSH enzyme activity	
Guven et al. 2009	rats			Increase in GSH enzyme activity	
Al-Dalain et al. 2001	rats				Ozone prevented oxidative stress damage
INHALED OZONE					
Forsberg et al -poster	3430				Ozone increases the amount of fibrinogen
Blomberg et al. 2003	22				Ozone increased serum Clara cell protein
Jakubowski et al. 2004	rats		Decreased levels		
Rudez et al. 2009	40		No effect		

During the past couple of decades, ozone has been wildly used in the medical field as ozone therapies, the most common being ozone autohemotherapy or ozonated autohemoadministration. However, its effects are still controversial. Advocates of these techniques sustain that ozone is beneficial for treating a large array of diseases including inflammatory and degenerative conditions of the bones and joints, cardiovascular diseases, reducing viral load in HIV infections and stopping cancer proliferation. At the other end of the spectrum, there are ozone treatments that resulted in the death of the patients or infections. The early techniques of injecting ozone gas into the patients veins, lead to pulmonary embolism and death of the patients. Most recently, the only fatality was caused by septicemia as a result of using contaminated needles and a more frequent effect was infection with hepatitis virus; however, these are not a consequence of ozone exposure, they are a result of improper administration of medical techniques.

It is common knowledge that ozone is a very strong oxidant, with a solubility 10-times higher than oxygen. Therefore, it would be expected that ozone would cause cell membrane damage, oxidative stress and inflammation. The reasons why it is so hard to assign ozone therapies to a definite class of effects can be explained by the “poison paradox: chemicals can behave as friends or foes depending on the dose and the biological system”. Taking it a step further, it is known that “most drugs produce many effects, all drugs produce at least two effects (Walsh, 2005). In conclusion, even though ozone is a strong oxidant, sometimes it can act as an antioxidant.

The objectives of this study are to improve the study design, used previously by other researchers, to determine optimal sample size and ozone concentration interval, to evaluate a

combination of inflammation and oxidative stress markers and to provide an answer to the controversy over the effects of ozone therapies.

The primary hypotheses tested were:

1. The concentration of C-reactive protein in the blood does not increase with increasing concentrations of ozone in blood.
2. Erythrocyte sedimentation rate is not affected by increasing concentrations of ozone in blood.
3. The ratio of reduced/oxidized glutathione does not change with increasing concentrations of ozone in blood.

The secondary hypotheses tested were:

1a. Ozone concentrations above 100 μg of ozone/ml of blood will increase the concentration of C- reactive protein in blood.

2a. Erythrocyte sedimentation rate increases with increasing concentrations of ozone in blood.

3a. The amount of oxidized glutathione increases with increasing concentrations of ozone in blood.

Significance of the research

This study does not approve or disapprove of the use of ozone therapies, but it does intend to shed some light onto the controversy that surrounds the health effects of ozone. The purpose of the study is to look at the effects of varying ozone concentrations that are within medical range (50-100 μg ozone/ml blood) compared to untreated blood, and the effects of ozone concentrations up to 3 times higher than the most common blood ozone therapy concentration, 50 μg ozone/ml blood and detect the concentration where ozone starts to have a deleterious effect on blood components that are primary markers of injury and/or inflammation. Previous studies analyzed different parameter combinations than the ones chosen in this study, and generally used only one ozone concentration and had a very small sample size consisting of patients with little variation in their health status. In general, autohemotherapy uses a concentration of 50 μg ozone /ml blood. Bagging techniques use higher concentrations, usually 100 μg ozone /ml air mixture.

Methods

Blood sample collection and preparation

The experiments were conducted on de-identified blood samples collected from the James A. Haley VA Hospital patients who were scheduled to have blood drawn by the Phlebotomy Lab staff. The sample size was set to include 20 patients and the blood from each patient was split into five subsamples, one to serve as a control and the other four to be treated with various ozone concentrations. The tests chosen to assess ozone damage were erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and glutathione ratio (GSH/GSSG). According to the study protocols used, the amounts of blood needed for each subsample were 1 ml blood for ESR, 150 µl blood for GSH/GSSG and 850 µl blood for CRP for a total of 2 ml/subsample and a total of 10 ml for a full set of experiments for each patient. Initially, the study design required each patient to donate 30 ml of blood, aside from the blood needed for the VA Hospital tests, in order to provide three replicates for each test. Following consultations with the VA Hospital and the IRB committee, it was decided that each patient will donate 10 ml of blood for this study. All patients who agreed to take part in this study were given information about the study, asked to acknowledge if they meet the exclusion criteria and then asked to sign a consent form. Because the parameters studied can be affected by certain inflammatory conditions, the exclusion criteria for the study were patients with HIV, hepatitis, rheumatoid arthritis,

pneumococcal meningitis, chronic lung disease, congestive heart failure, sickle cell anemia, polycythemia, inflammatory bowel disease and post-transplant patients. The blood was collected in tubes coated with EDTA to prevent blood coagulation.

Due to a high concern for the privacy of the patients it was not possible to persuade the IRB committee that a label of random sequences of numbers and letters would be de-identified enough to protect the privacy of the patients. As a result, in order to have to tubes labeled with random and unrelated codes, astronomical data for sun rising and setting as provided by the U.S. Navy Oceanography portal for different cities in the U.S. was used. None of these cities or data were associated with Tampa, FL or the James A. Haley VA Hospital. Once the blood was collected, the tubes were placed on ice and transported to the "Breath Lab" in the College of Public Health where the experiments were conducted. The ozone was produced by an ozone generator, using medical-grade oxygen. The ozone concentrations used in the study were 20, 40, 80 and 160 µg/ml of blood. Prior to exposing the blood to ozone, the OL80 ozone generator (from Ozone Services and Ozone Lab, Burton, BC, Canada) and the needle valve on the oxygen cylinder were calibrated for the concentrations used with a low flow rotameter and a high flow rotameter (see Appendix A, Table 3a, b and c).

Appropriate settings were developed from the calibration data to conduct the experiment. When using the low flow rotameter, the steel ball was chosen over the glass ball because it provided a better fit for the data, R^2= 0.975 compared to an R^2= 0.94. Similarly, when using the high flow rotameter, the steel ball was chosen over the glass ball because it provided a better fit for the data, R^2= 0.886 compared to an R^2= 0.865 (see Appendix A, Figure 1a and 1b).

The OLC80 ozone generator needed two different oxygen flows to generate the four ozone concentrations needed, 31 ml/min and 125 ml/min. Based on the two figures mentioned above it was determined that we needed to use the low flow rotameter on a setting of 79 for a flow rate of 31 ml/min and the high flow rotameter on a setting of 23 for a flow rate of 125 ml/min.

The next step was to calibrate the needle valve on the oxygen cylinder based on the rotameter settings. For the low flow rotameter the steel ball and a polynomial function provided a better fit for the data. For the high flow rotameter the steel ball and a polynomial function provided a better fit for the data. (see Appendix A, Figure 1c and 1d). Based on the above mentioned two figures, in order to obtain the flow rates needed by the ozone generator, the needle valve had to be positioned on a setting of 8 when using the low flow rotameter and a setting of 3.5 when using the high flow rotameter.

Using ozone-resistant syringes, 2 ml of blood were extracted from the 10 ml blood sample into each syringe, resulting in five syringes/blood sample or five syringes/patient. The five syringes corresponded to one control and four ozone treatments. The ozone generator and needle valve were set for the first ozone concentration used and the generator was allowed to run for five minutes to ensure that the concentration goal was reached. Then, a 2 ml volume of gas mixture at the desired concentration was extracted into the corresponding syringe, previously filled with blood, resulting in a 1:1 blood:gas mixture by volume ratio. The syringe was then placed on a platform mixer and allowed to mix 20 minutes at low speed to prevent foaming. This procedure was repeated for the other ozone concentrations. The control samples received 2 ml air and were then placed on the platform mixer.

Five blood samples were collected at one time for three out of the four collection days. During one of the collection days, one of the patients did not have enough blood to donate more than 5 ml of blood, therefore the sample had to be discarded and an additional patient enrolled in the study. The total sample size was twenty patients (n=20), each donating 10 ml of blood which was further divided into 2 ml subsamples. After the blood was divided into amounts specific for the three tests, the empty test tubes were disposed of appropriately, no later than 8 hours after blood collection.

Erythrocyte sedimentation rate (ESR)

After mixing, 1 ml of blood was taken from each syringe and inserted into the Wintrobe reservoir of its corresponding graduated tube for ESR. The graduated tube was inserted into the reservoir, adjusting its depth so the blood level reaches the "0" mm mark on the tube. The tubes had to be placed in a vertical position to prevent any bias in determining the sedimentation rate (see figure 2). One hour later, the difference between the blood level and the initial "0" mm level was determined. The difference, expressed as mm of blood/hour represents the erythrocyte sedimentation rate.

The erythrocyte sedimentation rates are affected by age and gender. Table 4 below shows the expected ESR values.

Table 4. Normal Erythrocyte Sedimentation Rates

(ESR 95% limits) mm/hr	**Age** (years)		
	20	**55**	**90**
Men	10	14	19
Women	15	21	23

All the tubes were discarded following ESR determination. The total number of tubes was 100, with five tubes for each of the twenty patients.

Figure 2. Wintrobe Tubes for Erythrocyte Sedimentation Rate.

C-reactive protein (CRP)

The C-reactive protein was assessed according to the Helica Biosystems research ELISA (enzyme-linked immunosorbent assay) protocol from blood serum. The serum was extracted from the 850 µl of whole blood left available from the 2 ml blood subsample. The blood was placed into microcentrifuge tubes and centrifuged to separate the serum. The serum was pipetted into fresh centrifuge tubes and frozen at -70ºC for one month, until CRP was determined. The total number of CRP samples evaluated was 100, which was five samples for each one of the twenty patients.

The reagents and the five standards were prepared according to the Helica protocol. The serum samples underwent a two-step dilution with wash buffer, the first at a 1:1,000 ratio and the second at a 1:4 ratio for a 1:4,000 total dilution. One hundred µl from each of the diluted serum samples was added in each of the corresponding microplate wells. The wells were coated with an affinity purified rabbit antihuman CRP-IgG. This was the antibody for human serum CRP (antigen). The microplates were then incubated at room temperature for 30 minutes to allow the samples to react with the antibody coating of the microplate wells. After the incubation, the microplates were washed four to five times with buffer (phosphate-buffered saline with Tween 20) and placed on paper towels to dry. Each well received 100 µl of conjugate, (a horseradish peroxidase (HRP)-labeled rabbit anti-human CRP-IgG with stabilizers and a preservative) followed by incubation and buffer washing as above. The purpose of the conjugate is to react with and tag the antigen-antibody complexes. After these steps, 100 µl of TMB (3,3', 5,5'-tetramethylbenzidine) were added in each well and allowed to

incubate for 10 minutes. If a blue color developed, that was an indication of a positive sample. Next, 100 µl of Stop solution (phosphoric acid) were added to each well. The stop solution causes the color to turn yellow, making it possible to be read in the microplate reader at 450 nm. Each microplate was run with a set of standard solutions at predetermined concentrations. A standard concentration curve was constructed using the absorbancy readings for each of the standards used (Table 5, Figure 3 in Appendix B).

Table 5. C-Reactive Protein Standards

Concentration (ng/ml)	Absorbance
3.33	1.292
3.33	1.355
1.11	0.561
1.11	0.517
0.37	0.234
0.37	0.234
0.12	0.122
0.12	0.151

The standard concentration curve was used to convert the absorbancy readings of the serum samples into C-reactive protein concentrations, multiplying by four to get the actual C-reactive protein serum concentration in µg/ml. The normal C-reactive protein levels are those between 0 and 5 µg/ml. However, some researchers consider 10 µg/ml to be the upper limit for normal CRP values. In this study, 5 µg/ml was the cut-off value used for C-reactive protein because a high risk of heart disease is associated with CRP values as low as of 3 µg/ml.

Higher levels of C-reactive protein are found during late pregnancy, mild inflammation and viral infections (10–40 mg/L), active inflammation, bacterial infection (40–200 mg/L) and severe bacterial infections and burns (>200 mg/L) (wikipedia.com).

Glutathione

The remaining 150 µl of blood from the original 2 ml blood sample treated with ozone were used to prepare the reduced (GSH) and oxidized (GSSG) glutathione samples. There were a total of 100 GSG and 100 GSSG samples, which were ten samples for each of the twenty patients.

Reduced glutathione is a tripeptide that contains a free thiol group. In a glutathione peroxidase catalized reaction, two molecules of GSH are bound together to form one molecule of GSSH.

For the accurate measurement of GSSG and the GSH/GSSG ratio, a glutathione assay needs to prevent the oxidation of GSH in the sample. In this case, a pyridine derivative was used as a thiol-scavenging reagent, which reacts quickly with GSH but does not interfere with the activity of the glutathione reductase enzyme. For a GSSG sample, thirty µl of thiol scavenger were added to a microcentrifuge tube then 100 µl of blood were added to the centrifuge tube and mixed gently. The purpose of the scavenger was to keep the glutathione in its oxidized form. For a GSG sample, 50 µl of blood were added into an empty centrifuge tube. All the samples were frozen at -70ºC until they were used for glutathione determination.

Prior to glutathione determination, the samples were thawed and prepared according to Oxford Biomedical GT-35 protocol as follows. Into the GSSG sample centrifuge tube were added 270 µl ice-cold 5% MPA (metaphosphoric acid), making a dilution factor x 4 and then the tube was vortexed briefly. The sample was then centrifuged at 1000 x *g* and 4°C for 10 minutes. After centrifuging, 50 µl of the

supernatant was collected with a pipette and added to 700 µL assay buffer in a new microcentrifuge tube. This step added to a dilution factor x 15 therefore making the total dilution factor x 60. The GSH sample centrifuge tube received 350 µL ice-cold 5% MPA, a dilution factor x 8 and was vortexed briefly. The sample was then centrifuged at 1000 x *g* and 4°C for 10 minutes. After centrifuging, 25 µl of the supernatant was collected with a pipette and added to 1.5 ml assay buffer in a new microcentrifuge tube. This step added to a dilution factor x 61 therefore making the total dilution factor x 488.

In parallel, all the reagents from the assay kit were reconstituted from received stock and assay buffer. The seven standard solutions used to make the calibration curve for GSG and GSSG were prepared (Table 6). Next, 200 µl of the blank solution (assay buffer) was added to a cuvette along with 200 µl DTNB (5,5'-Dithio-Bis 2-Nitrobenzoic Acid) solution and 200 µl reductase solution. The solutions in the cuvette were mixed and were incubated at room temperature for five minutes. After incubation 200 µl NADPH (nicotinamide adenine dinucleotide phosphate) solution added to the cuvette, causing the solution to turn yellow. The change of absorbance at 412 nm was recorded by taking readings every minute for 10 minutes. This procedure was repeated for all the standards and the GSH and GSSG samples.

Table 6. Glutathione Standards

Standard	µM GSH	µM GSSH	Rate	Net rate
B0	0.000	0.000	0	0
S1	0.100	0.050	0.0234	0.0234
S2	0.250	0.125	0.0293	0.0059
S3	0.500	0.250	0.0374	0.0315
S4	1.000	0.500	0.0588	0.0273
S5	1.500	0.750	0.068	0.0407
S6	2.000	1.000	0.0708	0.0301
S7	3.000	1.500	0.1018	0.0717

Because the concentration of GSSG is much lower in the reaction mixture compared to GSHt the protocol recommends that selected data ranges from the calibration curve should be plotted separately. For GSHt, linear regression was done on a three-point curve using the 0, 0.50, 0.75, 1.0, and 1.50 μM GSSG (0, 1.0, 1.5, 2.0, and 3.0 μM GSH) data points. In the case of GSSG, the 0, 0.05, 0.125, and 0.25 μM GSSG data points were used (see Appendix C, Figure 4).

After all the samples were read, an 11-point graph was generated for all samples; the slope of the line wass equal to sample rate. The calibration is described by the regression equation:

Net Rate = Slope x GSH + Intercept

In order to calculate the total GSH (GSHt) or GSSG concentration from the GSH calibration curve:

GSH =((Net Rate – Intercept)/slope) x Dilution Factor

The GSH/GSSG Ratio was calculated using the formula:

Ratio =(GSHt-2xGSSG)/GSSG

This assay measured the reduction of GSH to GSSG. The rate of the reaction was proportional to the GSH and GSSG concentration. The smaller the GSH/GSSG ratio, the higher the oxidative stress, as it would indicate a high amount of oxidized glutathione.

Statistical analysis

The data was tested for normality using leaf plots, box plots and normal probability plots. All three data sets were skewed to the left, indicating smaller numbers (smaller erythrocyte sedimentation rates, smaller concentrations of C-reactive protein, smaller glutathione ratios) were predominant.

SAS® statistical package and GLM procedure (General Linear Models) were used to detect differences among the ozone treatments and among the samples from the patients. The independent variables were the ozone treatments and the patients (samples). In the case of ESR, models using additional independent variables –age, gender, age nested within gender, interaction between age and gender were also used but only age was statistically significant. Least square means were computed for the independent variables, with p-values for differences in LS means.

Any difference with a probability $p < 0.05$ was considered statistically significant.

Results

Erythrocyte sedimentation rate

An indication of a positive effect of ozone therapies is a decrease in erythrocyte sedimentation rate. An increase in the erythrocyte sedimentation rate is a marker of inflammatory damage.

The erythrocyte sedimentation results observed in this study follow the pattern shown in previous studies of some positive effects, some negative effects, and some no effect. This is further complicated by the fact that some patients may exhibit both positive and adverse effects, depending on the ozone concentration used (see results in Table 7 below).

Table 7. Erythrocyte Sedimentation Rate Results

Erythrocyte Sedimentation Rate (mm/hr)					
Concentration *	0	1	2	3	4
Patient					
A	6	5	7.3	6.5	5
B	4	9	9	9	7
C	7	14	12.5	10	7.5
D	14	14	14	10	15
E	45	48	42	49	44
F	39	24	20	40	21
G	60	18	34	75	57
H	34	36	32	27	34
I	4	3	4	4	3
J	7	8	4	8	8
K	15	20	12	10	15
L	29	30	24	25	25
M	1	16	6	13	52
N	21	18	25	26	28
O	10	11	7	7	6
P	39	40	28	37	27
Q	48	32	39	29	18
R	7	8	9	8	9
S	40	24	18	16	40
T	1	4	7	4	4
*Where the ozone concentrations are: 0 =0 µg/ml, 1 =20 µg/ml, 2=40 µg/ml, 3= 80 µg/ml, 4= 160 µg/ml					

A decrease in erythrocyte sedimentation rate, a positive effect, was noticed in six out of the 20 patients. For the graphical representation see Appendix D, Figures 5c, 5l, 5o, 5p, 5q and 5s for patients C, L, O, P, Q, and S. The ages of these patients ranged between 31 and 73 years old. Out of these six patients, four of them showed a steadily decreasing trend in erythrocyte sedimentation rate from the control to the highest ozone concentration (Figure 5l, 5o, 5p and 5q). One of the six patients showed a decrease in erythrocyte sedimentation rate only for the lowest three ozone concentrations, 20 - 80 µg/ml (Figure 5s). The last of the six patients showed a decreasing trend in erythrocyte sedimentation rate from the lowest ozone concentration, 20 µg/ml to the highest ozone concentration 160 µg/ml but all these rates were higher than the baseline, 0 µg ozone/ml blood (Figure 5c).

An increase in erythrocyte sedimentation rate, which is an adverse effect as it indicates an inflammatory condition was noticed in four out of the 20 patients. These results are shown in Appendix B, Figures 5g, 5m, 5n and 5s for patients G, M, N, and S. The ages of these patients ranged from 29 to 63 years old. Two of these four patients showed an increasing trend from the control to the highest ozone concentration (Figure 5m and 5n). One of the four patients showed an increase in the erythrocyte sedimentation rate from the lowest ozone concentration to the highest but the results for the two lowest ozone concentrations were still better than the patient's baseline (Figure 5g). The last of the four patients showed an increase in erythrocyte sedimentation rate only for the highest ozone concentration (Figure 5s).

A total of eleven patients showed no effect on the erythrocyte sedimentation rate when comparing the control and the four ozone concentrations (Figure 5a, 5b, 5d, 5e, 5f,

5h, 5i, 5j, 5k, 5r, and 5t for patients A, B, D, E, F, H, I, J, K, R, and T). The ages of these patients cover the entire spectrum from 22 to 74 years old.

When plotting the results for controls only (Figure 6 below), age does not appear to have an influence on erythrocyte sedimentation rate. An equal number of people below and over 50 years old have normal erythrocyte sedimentation rates.

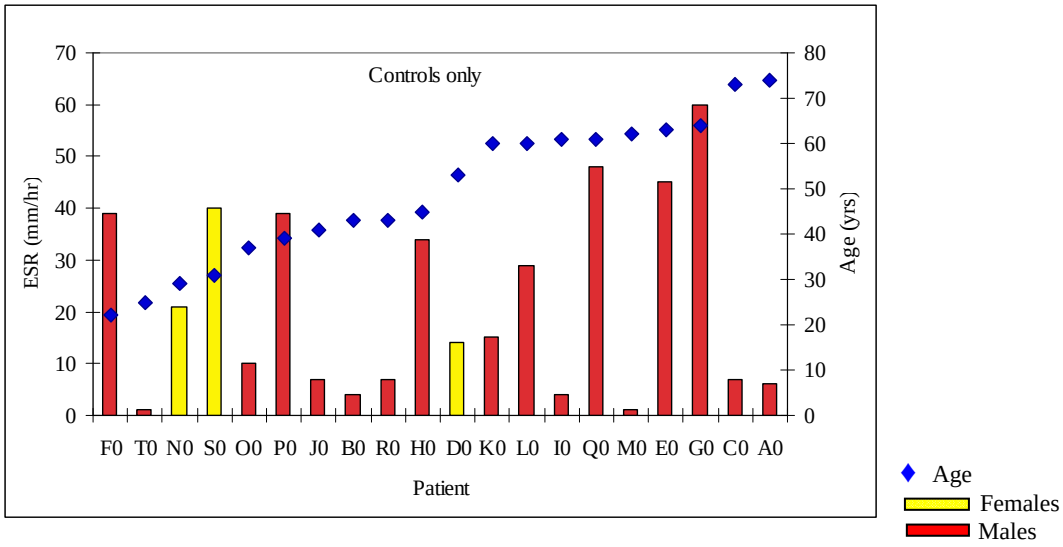


Figure 6. Erythrocyte Sedimentation Rates for Controls. The patients are denoted by letters from A to T, arranged based on their age and the number 0 next to the patient identification letter represents the baseline, 0 µg ozone/ml blood.

Similarly, when plotting the results for the treatments only (Figure 7 below), people below and over 50 years old had equal numbers of erythrocyte sedimentation rates above the normal limit.

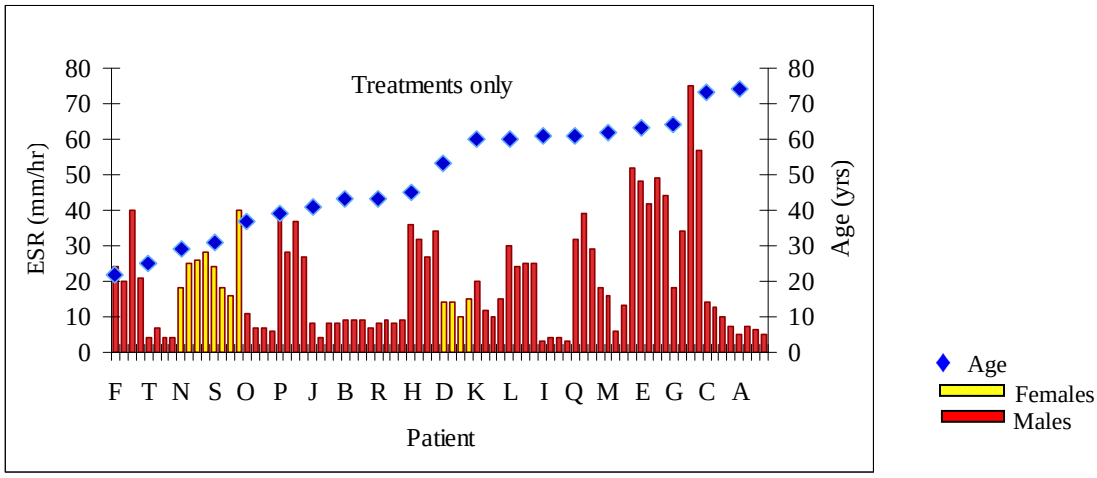


Figure 7. Erythrocyte Sedimentation Rates for Ozone Treatments. The patients are denoted by letters from A to T, arranged based on their age with the four bars/patient representing each ozone concentration in ascending order.

A closer look at the combined effects of ozone and age reveals that people below 40 years old generally showed a decrease in erythrocyte sedimentation rate with increasing ozone concentrations. This is a positive result. In contrast, people above 45 years old had an increase in erythrocyte sedimentation rate under the four ozone concentrations compared to the control. This is an adverse result, indicating an inflammatory response (Figure 8a and b).

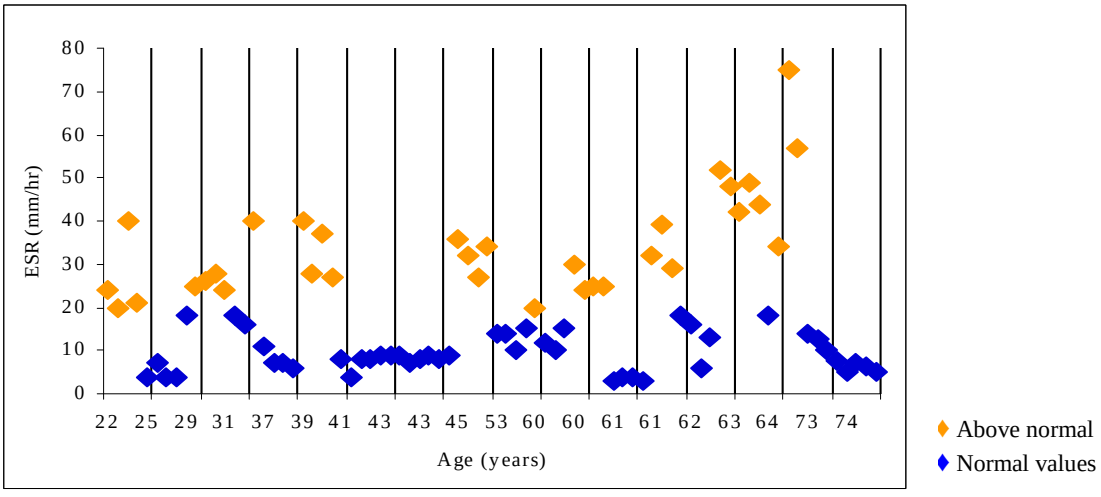


Fig. 8a. comparison between normal and above normal values

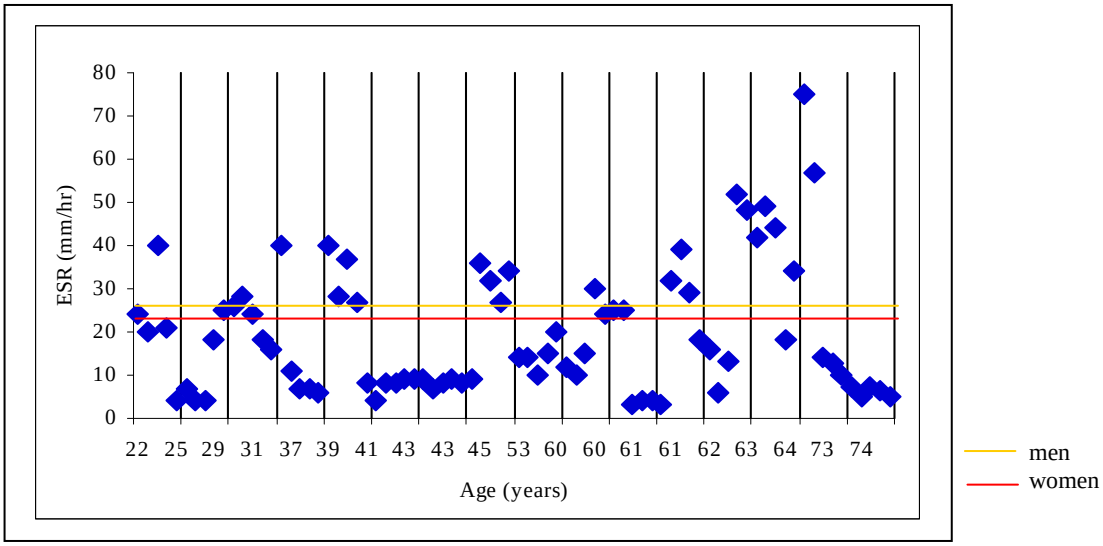
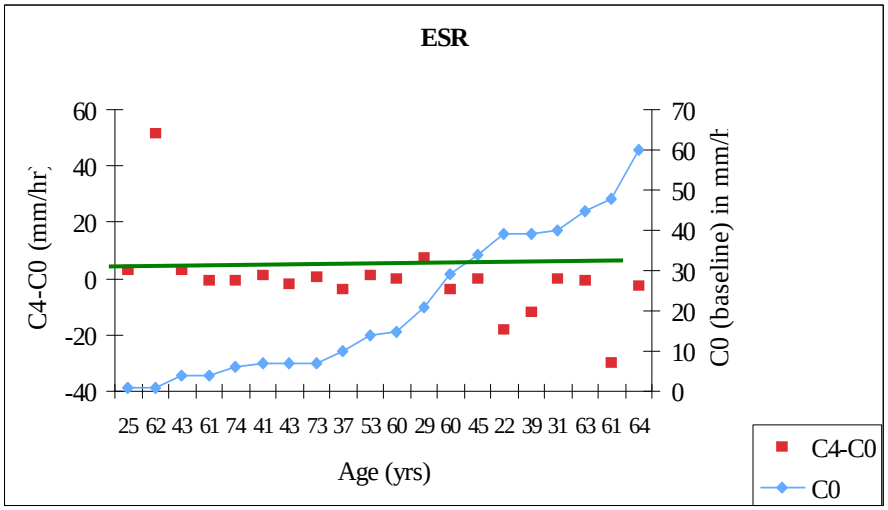


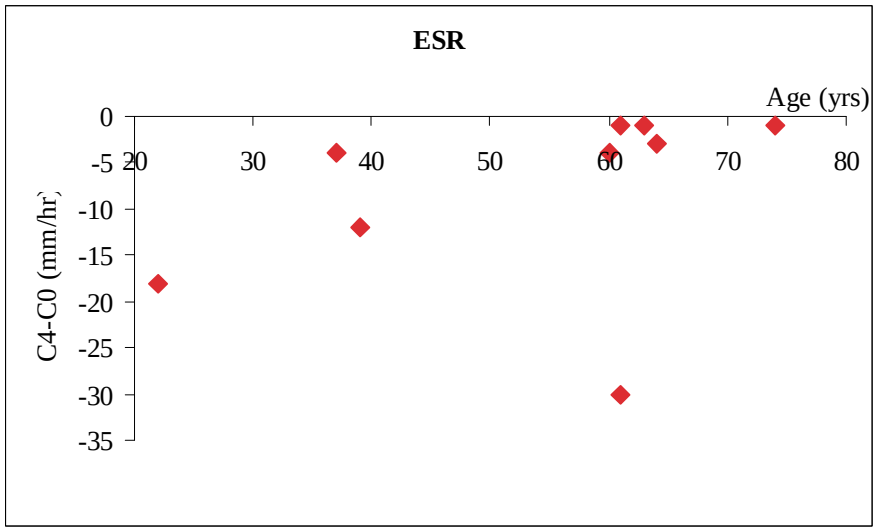
Fig. 8b. normal values for men and women

Figure 8. Combined Erythrocyte Sedimentation Rates.

Next, the results for the erythrocyte sedimentation rate obtained at the highest ozone concentration, C4=160 µg/ml, were compared to the baseline (C0) erythrocyte sedimentation rate (Figure 9a). A negative difference between the erythrocyte sedimentation rate at the highest ozone concentration and the one at baseline indicates a decrease in the erythrocyte sedimentation rate due to ozone treatment, indicating a positive result (shown in Figure 9b). Most of the patients who showed a negative C4-C0 difference were over 60 years old, indicating that they are the category that responds best to high ozone concentrations.



a. Values above show a positive (C4-C0) difference, values below show a negative difference



b. Nine of the 20 patients showed a negative C4-C0 difference

Figure 9. Difference in Erythrocyte Sedimentation Rate between the Highest Ozone Treatment and Baseline

When the same comparison was done between the lowest ozone concentration C1=20 µg/ml and the baseline, C0 a similar number of people under and over 60 years

old had negative C1-C0 differences indicating a decrease in erythrocyte sedimentation rate as a result of ozone treatment (Figure 10).

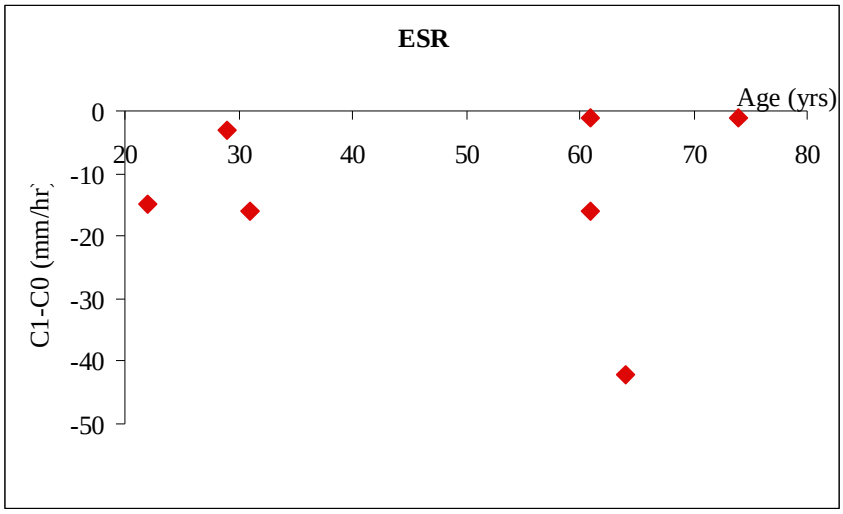


Figure 10. Difference in Erythrocyte Sedimentation Rate Between the Lowest Ozone Treatment and Baseline

However, the statistical models show that there are no statistically significant differences among the ozone concentrations, p=0.56. The age of the patients and the differences between patients are statistically significant, both with p=0.0001 but for the ozone concentrations used the statistical model could not detect a difference among ozone treatments.

C-reactive protein

Similar to the erythrocyte sedimentation rate, an indication of a positive effect of ozone therapies is also a decrease in the concentration of C-reactive protein. An increase in C-reactive protein is a marker of inflammatory damage.

Again, the C-reactive protein results observed in this study follow the pattern shown in previous studies of some positive effects, some negative effects, and some showing no effect. This is further complicated by the fact that some patients may exhibit both positive and adverse effects, depending on the ozone concentration (see results in Table 8).

Table 8. C-Reactive Protein Results

C-reactive protein (mg/l)					
Concentration	0	1	2	3	4
Patient					
A	0.87	0.51	0.59	0.64	0.66
B	0.73	0.92	2.23	1.69	3.72
C	3.14	2.94	4.36	2.16	4.91
D	0.79	0.48	0.55	0.61	1.34
E	3.99	1.62	0.98	2.94	0.92
F	0.56	0.61	0.42	1.25	0.68
G	2.21	1.72	0.59	1.02	0.66
H	0.71	1.90	0.79	1.09	0.56
I	3.86	1.83	0.69	1.71	0.6
J	0.87	2.64	0.63	1.15	0.83
K	0.92	0.92	0.66	0.82	0.98
L	0.77	1.44	1.2	1.11	0.68
M	12.35	0.93	0.93	4.27	11.45
N	7.71	6.83	5.03	0.77	0.9
O	1.26	0.42	0.57	0.44	0.47
P	0.61	0.72	0.45	0.5	0.49
Q	1.73	0.56	0.54	0.5	0.56
R	0.42	0.44	0.41	0.45	0.56
S	0.99	0.78	0.48	0.58	0.48
T	0.43	0.44	0.49	0.58	1.09
Where the ozone concentrations are: 0 =0 µg/ml, 1 =20 µg/ml, 2=40 µg/ml, 3= 80 µg/ml, 4= 160 µg/ml					

A decrease in C-reactive protein, which is a positive effect, was noticed in five out of the 20 patients. For the graphical representation see Appendix E, Figures 11e, 11g, 11i, 11m, and 10n for patients E, G, I, M, and N. With the exception of patient N the age of these five patients ranged between 61 and 64 years old. Out of these five patients, four of them showed a steadily decreasing trend in C-reactive protein from the control to the highest ozone concentration (Figure 11e, 11g, 11i, and 11n). One of the five patients, patient M showed a decrease in C-reactive protein only for the lowest two ozone concentrations, 20, respectively 40µg/ml (Figure 11m).

An increase in C-reactive protein was observed in two out of the 20 patients (Figure 11b and previous 11m for patients B and M). The cut-off value used, between normal and abnormal C-reactive protein concentrations was of 5 mg/ml. Patient M showed previously a decrease in C-reactive protein for the lowest two ozone concentrations, with the C-reactive protein increasing for the highest two ozone concentrations, 80, respectively 160 µg/ml. This would be interpreted as a negative result, however, even with the increase in C-reactive protein these levels were lower than the baseline.

Overall, fourteen out of the 20 patients showed no change in the C-reactive protein concentration as a results of ozone treatments.

When the results for the 20 patients are combined, it becomes obvious that most of the patients had normal C-reactive protein values, except for 3 patients, two of which had elevated values only for the highest ozone concentration treatment. The other patient had elevated values for the lowest two ozone concentrations (Figure 12).

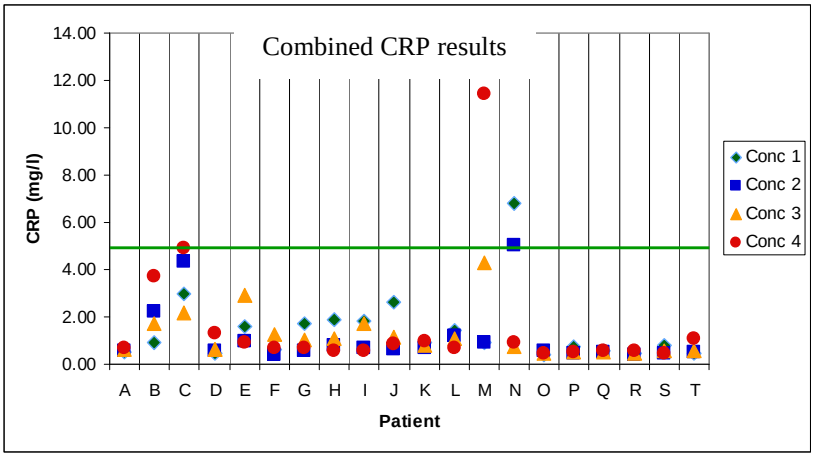


Figure 12. Combined C-Reactive Protein Results

The statistical models showed that there are no statistically significant differences among the ozone concentrations, p=0.177. Only the differences between patients are statistically significant, with p=0.0001.

Glutathione

An indication of a positive effect of ozone therapies is an increase in the reduced to oxidized glutathione ratio. A decrease in the reduced to oxidized glutathione ratio is a marker of oxidative stress.

Similar to erythrocyte sedimentation rate and C-reactive protein, the glutathione ratio results observed in this study follow the pattern shown in previous studies of a combination of positive, negative effects, and no effects (see Table 9 below). This is further complicated by the fact that some patients may exhibit both positive and adverse effects, depending on the ozone concentration.

Table 9. Glutathione Ratio Results

GSH/GSSG ratio					
Concentration	0	1	2	3	4
Patient					
A	1.95	1.92	2.47	1.87	2.92
B	1.97	2.47	1.87	2.09	1.97
C	2	2.05	2.01	1.99	2.01
D	2.04	1.96	1.99	1.97	1.95
E	2.17	1.98	1.3	2.37	1.98
F	2.04	1.68	1.64	1.7	2.28
G	2.04	1.68	1.64	1.7	2.28
H	0.16	1.75	1.97	1.9	4.6
I	0.99	1.65	1.88	1.86	1.89
J	1.73	2.05	2.1	2.34	2.75
K	1.85	1.44	2.4	2.21	3.77
L	2.01	2.59	1.48	2.21	3.27
M	1.28	1.6	1.86	1.87	2.13
N	3.13	2.42	1.72	1.68	1.91
O	1.37	1.68	1.98	1.21	0.96
P	1.86	1.82	1.85	1.84	2.08
Q	1.93	1.34	0.63	1.55	0.81
R	2.16	1.45	3.51	3.32	2.65
S	2.5	2.08	2.49	2.22	2.66
T	2.38	2.68	2.14	2.82	2.83
Where the ozone concentrations are: 0 =0 µg/ml, 1 =20 µg/ml, 2=40 µg/ml, 3= 80 µg/ml, 4= 160 µg/ml					

An increase in the glutathione ratio, a positive effect, was noticed in six out of the 20 patients. For the graphical representation see Appendix F, Figures 13a, 13f, 13h, 13k, 13l, and 13r for patients A, F, H, K, L, and R. These patients ranged in age from 22 to 74 years old. One of the six patients, F, experienced an increase in the glutathione ratio only for the two highest ozone concentrations, the two lowest ozone concentrations having an adverse effect (Figure 13f).

A decrease in the glutathione ratio, which is an adverse effect, was noticed in four out of the 20 patients (Figure 13b, 13f, 13o, and 13q for patients B, F, O, and Q. with ages ranging from 22 to 61 years old). Out of these four patients, one showed a decrease in the glutathione ratio only for the highest two ozone concentrations (Figure 13o) while another showed a decrease in the glutathione ratio only for the lowest two ozone concentrations (Figure 13f).

A total of ten patients showed no effect on the glutathione ratio when comparing the control and the four ozone concentrations (Figure 13d, 13e, 13g, 13i, 13j, 13m, 13n, 13p, 13s, and 3t for patients D, E, G, I, J, M, N, P, S, and T). The ages of these patients cover the entire spectrum from 25 to 64 years old.

The statistical models showed that there are no statistically significant differences among the ozone concentrations, $p=0.2379$ and among the patients, $p=0.2495$.

Combined results

The response of the patients to the three tests was combined in Table 10 below. The responses were coded with numbers, 0 meaning no effect, positive numbers meaning an increase in the parameter tested and negative numbers a decrease in the parameter tested.

A decrease erythrocyte sedimentation rate or C-reactive protein as a result of ozone treatment is a desired positive effect while for glutathione, an increase in the GSH/GSSH ratio as a result of ozone treatment is a positive effect.

Based on their score for the three tests combined, the patients were assigned into categories from I to V with I indicating the highest overall change and V meaning no change (see legend below).

Legend:

-2 decreasing	I highest change for all 3 tests
-1 slightly decreasing	II high change across all 3 tests
0 unchanged	III moderate change across all 3 tests
+1 slightly increasing	IV least change across all 3 tests
+2 increasing	V no change

Table 10. Patient Response to all Three Tests

PATIENT			TEST			
	Gender	Age	ESR	CRP	GSH/GSSG ratio	Overall change
A	M	74	0	0	+1	IV
B	M	43	0	+2	-1	II
C	M	73	0	+1	n/a	IV
D	F	53	0	0	0	V
E	M	63	0	-2	0	III
F	M	22	0	0	0	V
G	M	64	+1	-1	0	III
H	M	45	0	0	+1	IV
I	M	61	0	-2	0	III
J	M	41	0	0	0	V
K	M	60	0	0	0	V
L	M	60	0	0	+2	III
M	M	62	+2	+2*	0	I
N	F	29	+1	-2	0	II
O	M	37	0	0	0	V
P	M	39	-1	0	0	IV
Q	M	61	-2	0	-1	II
R	M	43	0	0	+1	IV
S	F	31	-1	0	0	IV
T	M	25	0	0	+1	IV

*INCREASE AMONG OZONE CONCENTRATIONS, CONTROL EXCLUDED

Most patients (12 patients) experienced little to no change to this three tests. Four of the patients showed moderate change across all three tests. Three patients showed high change across all three tests and only one was extremely responsive to the three tests. The patient with the highest responsiveness had the highest increase in erythrocyte sedimentation rate compared to the control and C-reactive protein concentration

compared to the lowest two ozone concentrations. The patients with high change mostly had negative changes as a result of ozone treatment while the patients with moderate changes showed positive improvements.

Discussion

The main purpose of this study was to determine if ozone therapies are harmful for the samples and should not be offered as alternative treatment techniques in hospitals.

Based on the parameters studied, ozone therapies would have a harmful effect if the samples experienced a statistically significant increase in erythrocyte sedimentation rate and C-reactive protein concentration and a statistically significant decrease in the glutathione ratio. Ozone therapies typically use an ozone concentration of 50 µg of ozone/ml blood. Ozone bagging techniques utilize concentrations up to 100 µg of ozone/ml air mixture. The range of ozone concentrations used in this study were chosen to cover the therapeutical spectrum and ranged from 20 µg of ozone/ml blood to160 µg/ml.

Our results were not able to reject the primary hypotheses and infer that the ozone therapies are harmful. While most of the samples did not have any reaction to the ozone treatments, about forty percent of the samples had either a positive or negative response. Overall, 30 % of the samples experienced a decrease in erythrocyte sedimentation rate for the four ozone concentrations, which is a positive effect of ozone treatments. If the ozone concentrations were studied individually, the most efficient ozone concentration was 40 µg/ml, with 55% of the samples showing a decrease in erythrocyte sedimentation rate. When the blood was treated with a concentration of 160 µg/ml, 45% of the samples showed a decrease in erythrocyte sedimentation rate. A concentration of 80 µg/ml caused

an erythrocyte sedimentation rate decrease in 40 % of the samples while a concentration of 20 µg/ml was least efficient, with 35 % of the samples showing a decrease in erythrocyte sedimentation rate. If the samples are evaluated individually, 20 % of them had the highest decrease in the erythrocyte sedimentation rate at the 80 µg/ml ozone concentration, 15 % at 40 µg/ml and 160 µg/ml and 10 % at 20 µg/ml.

A negative response to the four ozone treatments was found in 20 % of the samples. Sixty percent of the samples had a decrease in erythrocyte sedimentation rate at 20 µg/ml, 55 % of the samples at 80 µg/ml, 40 % at 160 µg/ml and 35 % at 40 µg/ml.

An overall decrease in the C-reactive protein for each of the four ozone concentrations was noticed in 25 % of the samples (changes less than 0.05 mg/l were ignored). However, individual ozone concentrations had a bigger impact than their combined effect. Most samples showed a positive effect at a particular ozone concentration only, while the other concentrations had no effect. The most efficient ozone concentration was again 40 µg/ml, with 75% of the samples showing a decrease in C-reactive protein. Concentrations of 160 µg/ml and 80 µg/ml caused a decrease in C-reactive protein in 65 % of the samples. The lowest ozone concentration, 20 µg/ml determined a decrease in C-reactive protein in 55 % of the samples. When the samples are evaluated individually, 35 % of them had the highest decrease in C-reactive protein at the 40 µg/ml ozone concentration, 20 % at 20 µg/ml and 160 µg/ml and 10 % of the samples at 80 µg/ml.

A negative C-reactive protein response to the four ozone treatments was noticed in 10 % of the samples. Each of the two highest ozone concentrations, 80 and 160 µg/ml, and the lowest ozone concentration, 20 µg/ml caused a negative response in 30 % of the

samples. The 40 µg/ml concentration caused a negative response in 15 % of the samples. Individual results showed 30 % of the samples had the highest negative response at the highest ozone concentration, 160 µg/ml, 20 % of the samples at the lowest ozone concentration, 0.2 µg/ml and 10 % at the 80 µg/ml œncentration.

For the glutathione ratio, a positive effect was represented by an increase in the ratio as a result of exposure to ozone (changes less than 0.1 were ignored). Thirty % of the samples had a positive response for all ozone concentrations. Some samples responded to only one particular ozone concentration. The highest ozone concentration, 160 µg/ml proved to be the most effective with 65 % of the samples experiencing an increase in the glutathione ratio. This was followed by the 80 µg/ml concentration with 50 % of the samples and the lowest ozone concentrations each with 40 % of the samples. When the samples are evaluated individually, 60 % of them had the highest increase in the glutathione ratio at the 160 µg/ml ozone concentration, 10 % at 20 µg/ml and 40 µg/ml and 5 % of the samples at 80 µg/ml.

A negative glutathione ratio response to the four ozone treatments was noticed in 20 % of the samples. Each of the two lowest ozone concentrations, 20 and 40 µg/ml, caused a negative response in 40 % of the samples, followed by the 80 µg/ml concentration with 30 % of the samples and 160 µg/ml with 20 % of the samples. When the samples are evaluated individually, 10 % of them had the highest increase in the glutathione ratio at the 160 µg/ml ozone concentration, respectively 80 µg/ml, 25 % at 20 µg/ml and 35 % of the samples at 40 µg/ml.

Most of the samples showed none to little response to ozone treatments for the three tests studied, as it was reinforced by the lack of statistical significance when the

differences between treatments were evaluated. The statistical models as a whole, which included the samples as the independent variable and their age and gender (for erythrocyte sedimentation rate) were statistically significant. However the results for the three tests were not able to determine a difference between the ozone concentrations used. This means that the study could not prove that ozone therapies are harmful, even though some of the concentrations used were significantly higher than the therapeutical concentrations.

However, most of the samples (12) showed an improvement in one of the tests. This suggests that ozone therapies can be used to improve the general health status of a patient. Even though the ozone therapies may not have the potency to cure cancer or AIDS, they could be a very useful tool if they are used as an adjuvant for established medical procedures if their effect is additive. The results obtained in this study demonstrate the difficulty that previous studies had in pinpointing an effect of ozone therapies. It appears clear that the ozone therapies have to be tailored for the individual as there is no general formula for their effectiveness. It is also possible that the blood may not be an appropriate media for testing the effect of ozone therapies as the blood antioxidant capacity may be able to counteract some of these effects.

For future research, it would be very useful to develop a better test, using parameters that correlate with ozone therapy concentrations, with a greater sensitivity and lower variability for the data.

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Appendix A

Calibration Data for the Rotameters and Needle Valve

Appendix A (Continued)

Table 3. Calibration Data for the Rotameters and Needle Valve

a. Low Flow (0-0.05 l/min) Rotameter

CALIBRATION OF LOW FLOW ROTAMETER- GLASS BALL						
SETTING	VOLUME	TIME	TIME	FLOW RATE	SETTING	FLOW RATE
	CC	SECONDS	MINUTES	CC/MIN		ML/MIN
51	5	57	0.95	5.3	46	5.3
46	5	59	0.98	5.1	51	5.3
101	5	24	0.40	12.5	94	12.5
98	5	25	0.42	12.0	96	12.0
96	5	27	0.45	11.1	98	11.1
94	5	27	0.45	11.1	101	11.1
117	5	19	0.32	15.8	111	15.8
115	5	21	0.35	14.3	113	14.3
113	5	20	0.33	15.0	115	15.0
111	5	21	0.35	14.3	117	14.3
140	5	14	0.23	21.4	138	21.4
139	5	15	0.25	20.0	139	20.0
138	5	15	0.25	20.0	140	20.0

CALIBRATION OF LOW FLOW ROTAMETER- STAINLESS STEEL BALL						
SETTING	VOLUME	TIME	TIME	FLOW RATE	SETTING	FLOW RATE
	CC	SECONDS	MINUTES	CC/MIN		ML/MIN
14	5	57	0.95	5.3	12	5.26
12	5	59	0.98	5.1	14	5.08
38	5	24	0.40	12.5	34	12.50
36	5	25	0.42	12.0	35	12.00
35	5	27	0.45	11.1	36	11.11
34	5	27	0.45	11.1	38	11.11
47	5	19	0.32	15.8	44	15.79
46	5	21	0.35	14.3	45	14.29
45	5	20	0.33	15.0	46	15.00
44	5	21	0.35	14.3	47	14.29
68	5	14	0.23	21.4	62	21.43
66	5	15	0.25	20.0	66	20.00
62	5	14	0.23	21.4	68	21.43
110	5	7	0.12	42.9	105	42.86
109	5	8	0.13	37.5	108	37.50
108	5	7	0.12	42.9	109	42.86
105	5	7	0.12	42.9	110	42.86
136	5	5	0.08	60.0	135	60.00
136	5	5	0.08	60.0	136	60.00
136	5	5	0.08	60.0	136	60.00
135	5	5	0.08	60.0	136	60.00

Appendix A (Continued)

Table 3 (Continued)
b. High Flow (0.05-0.8 l/min) Rotameter

CALIBRATION OF HIGH FLOW ROTAMETER						
GLASS BALL						
SETTING	VOLUME	TIME	TIME	FLOW RATE	SETTING	FLOW RATE
	CC	SECONDS	MINUTES	CC/MIN		ML/MIN
27	5	6	0.10	50.0	27	50.00
27	5	6	0.10	50.0	27	50.00
27	5	6	0.10	50.0	27	50.00
27	5	7	0.12	42.9	27	42.86
27	5	7	0.12	42.9	27	42.86
27	5	7	0.12	42.9	27	42.86
40	5	7	0.12	42.9	39	42.86
40	5	4	0.07	75.0	39	75.00
40	5	5	0.08	60.0	39	60.00
39	5	5	0.08	60.0	39	60.00
39	5	5	0.08	60.0	40	60.00
39	5	4	0.07	75.0	40	75.00
39	5	5	0.08	60.0	40	60.00
60	5	5	0.08	60.0	60	60.00
60	5	3	0.05	100.0	60	100.00
60	5	3	0.05	100.0	60	100.00
60	5	3	0.05	100.0	60	100.00
60	5	3	0.05	100.0	60	100.00
60	5	3	0.05	100.0	60	100.00
97	5	3	0.05	100.0	97	100.00
97	5	2	0.03	150.0	97	150.00
97	5	2	0.03	150.0	97	150.00
97	40	11	0.18	218.2	97	218.18
97	40	11	0.18	218.2	97	218.18
146	30	5	0.08	360.0	146	360.00
146	40	6	0.10	400.0	146	400.00

Appendix A (Continued)

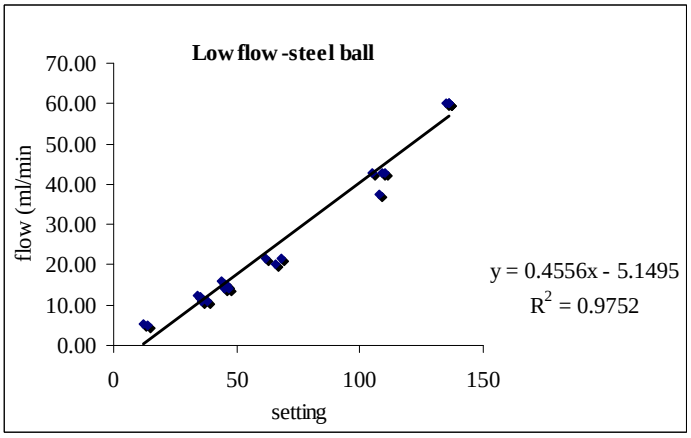
Table 3b. (Continued)

CALIBRATION OF HIGH FLOW ROTAMETER						
STAINLESS STEEL BALL						
SETTING	VOLUME	TIME	TIME	FLOW RATE	SETTING	FLOW RATE
	CC	SECONDS	MINUTES	CC/MIN		ML/MIN
2	5	6	0.10	50	2	50.00
2	5	6	0.10	50	2	50.00
2	5	6	0.10	50	2	50.00
2	5	7	0.12	43	2	42.86
2	5	7	0.12	43	2	42.86
2	5	7	0.12	43	2	42.86
2	5	7	0.12	43	2	42.86
10	5	5	0.08	60	10	60.00
10	5	5	0.08	60	10	60.00
10	5	4	0.07	75	10	75.00
10	5	5	0.08	60	10	60.00
10	5	4	0.07	75	10	75.00
10	5	5	0.08	60	10	60.00
10	5	5	0.08	60	10	60.00
24	5	3	0.05	100	24	100.00
24	5	3	0.05	100	24	100.00
24	5	3	0.05	100	24	100.00
24	5	3	0.05	100	24	100.00
24	5	3	0.05	100	24	100.00
24	5	3	0.05	100	24	100.00
44	5	2	0.03	150	44	150.00
44	5	2	0.03	150	44	150.00
44	5	1	0.02	300	44	300.00
44	40	11	0.18	218	44	218.18
44	40	11	0.18	218	44	218.18
69	30	5	0.08	360	69	360.00
69	40	6	0.10	400	69	400.00

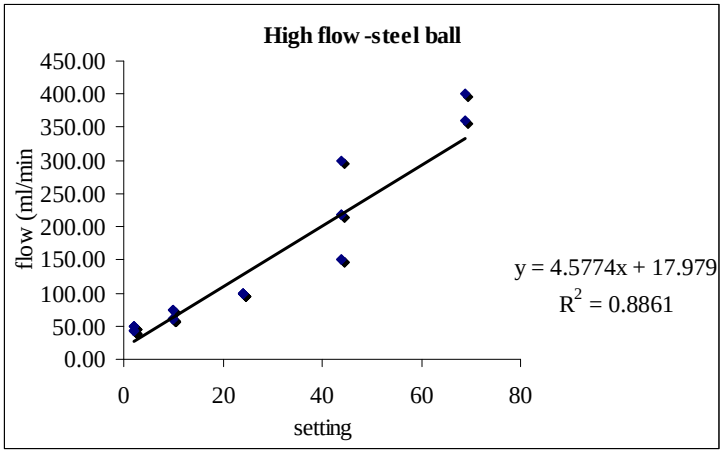
Appendix A (Continued)

Table 3 (Continued)
c. Calibration of the Needle Valve for the Low Flow and High Flow Rotameters

Low flow rotameter			High flow rotameter		
	Setting			Setting	
Needle valve	Glass	Steel	Needle valve	Glass	Steel
setting	ball	ball	setting	ball	ball
6.5	0.0	5	2	0	8
6.5	0.0	6	2	0	11
6.5	0.0	6	2	0	11
6.5	0.0	6	4	0	25
7	0.0	9	4	0	25
7	0.0	10	4	0	26
7	0.0	10	6	11	41
7	0.0	10	6	11	41
7.5	0.0	11	6	11	41
7.5	0.0	11	8	29	69
7.5	0.0	12	8	29	69
7.5	0.0	12	8	29	69
8	18.0	73	10	33	84
8	30.0	74	10	37	86
8	21.0	80	10	36	86
8	24.0	85			
8.5	71.0	140			
8.5	73.0	143			
8.5	74.0	143			
8.5	66.0	150			

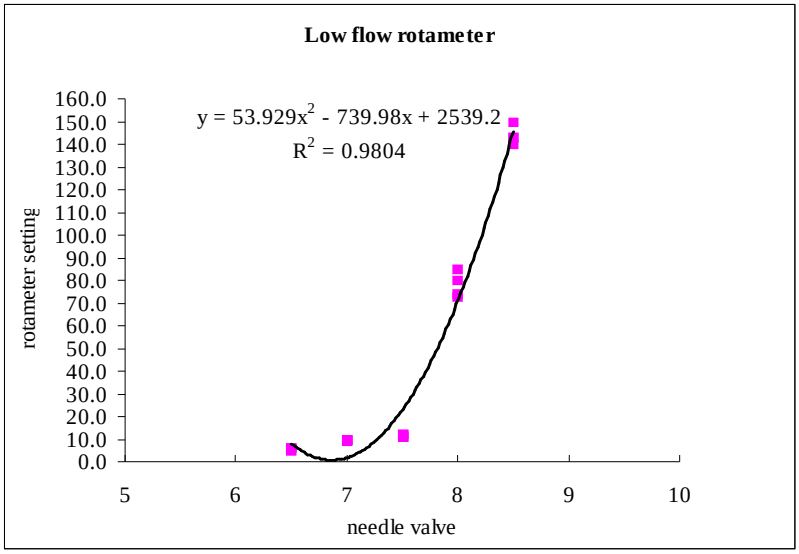


a. Calibration of the low flow (0-0.05 l/min) rotameter using the steel ball.

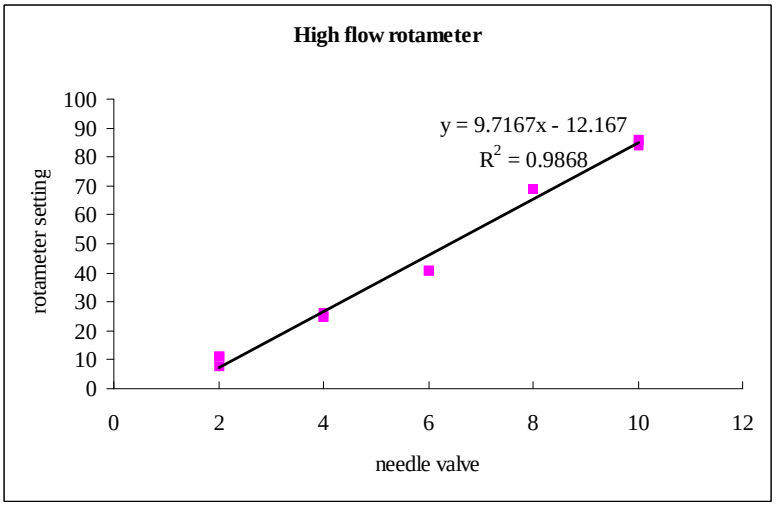


b. Calibration of the high flow (0.05-0.8 l/min) rotameter using the steel ball.

Appendix A (Continued)



c. Calibration of the needle valve for the low flow rotameter.



d. Calibration of the needle valve for the high flow rotameter.

Figure 1. Calibration Data for the Rotameters and Needle Valve

Appendix B

Figure 3. Calibration Curve for C-Reactive Protein

Appendix B (Continued)

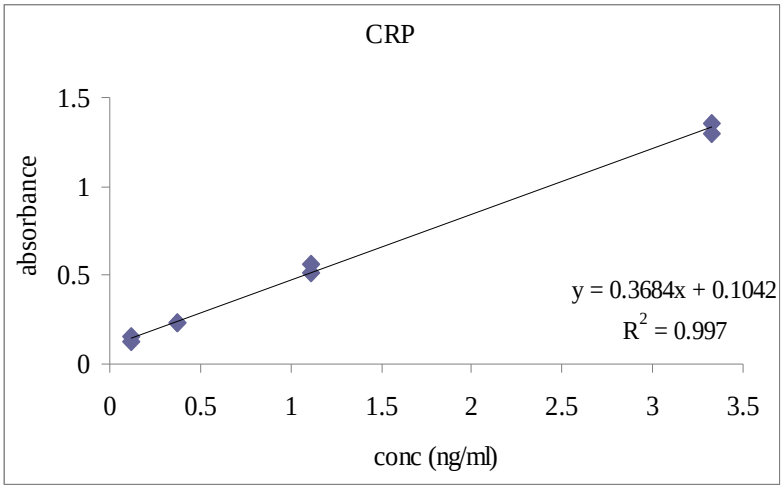
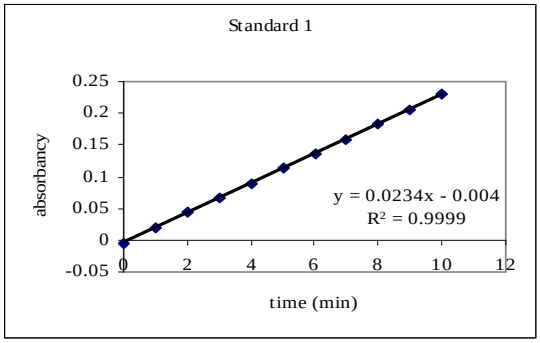


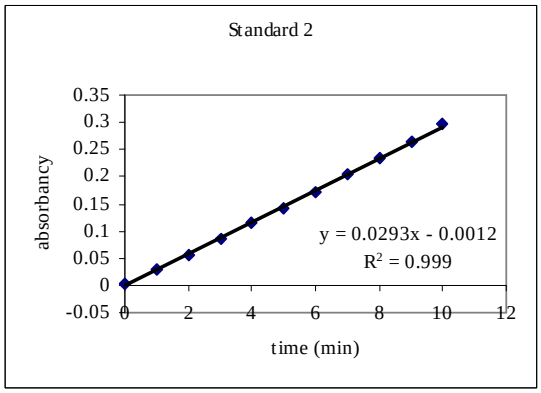
Figure 3. Calibration Curve for C-Reactive Protein

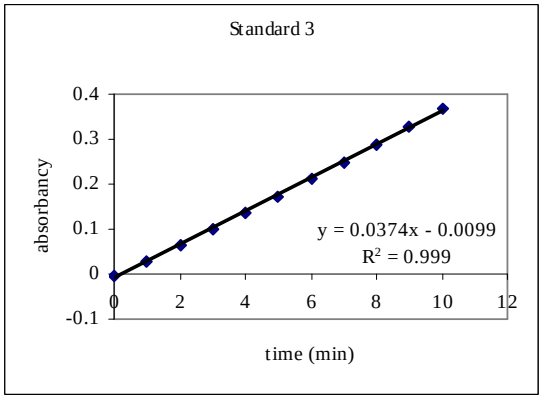
Appendix C

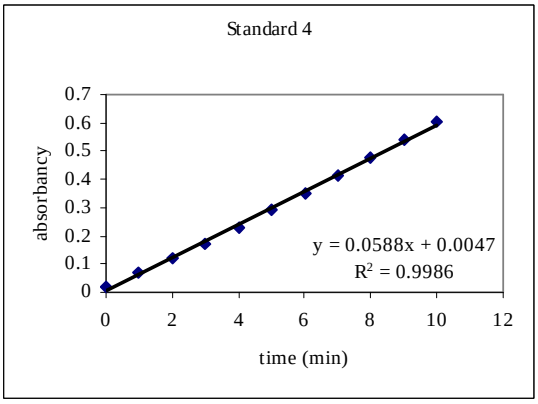
Figure 4. Calibration Curves for Glutathione

Appendix C (Continued)

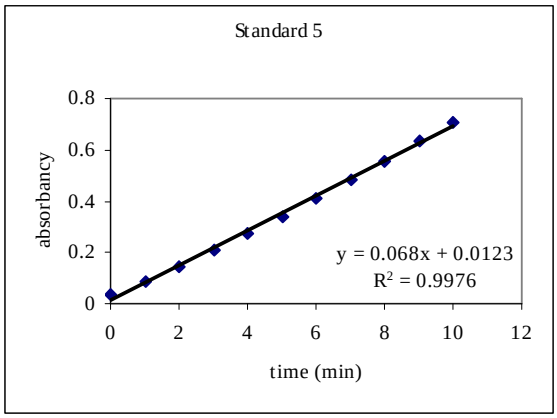


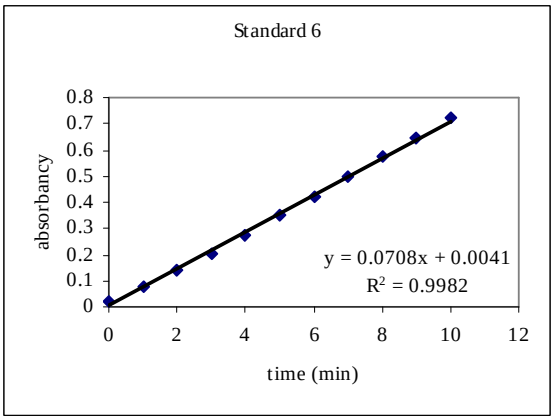


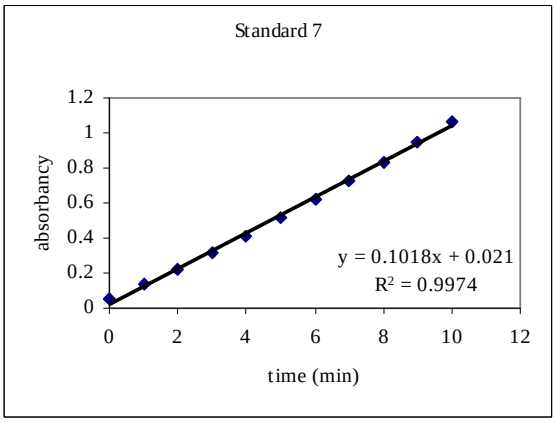




Appendix C (Continued)







Appendix D

Figure 5. Erythrocyte Sedimentation Rates

Note: In the following figures, the capital letter denotes the patient (from A to T), the 0-4 numbers represent the ozone concentration (4 being the highest ozone concentration), the small letter ‘s’ denotes a GSH sample and the double letter ‘ss’ denotes a GSSG sample. The green line indicates a positive effect and the red line an adverse effect.

Appendix D (Continued)

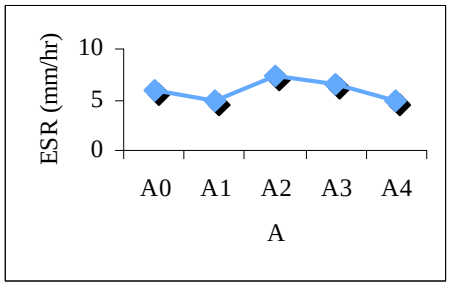


Fig. 5a.

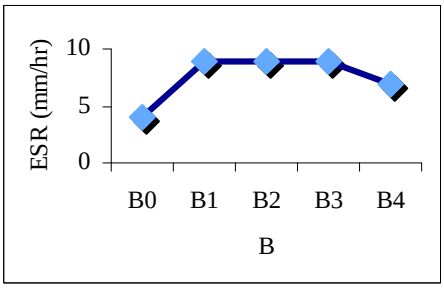


Fig. 5b.

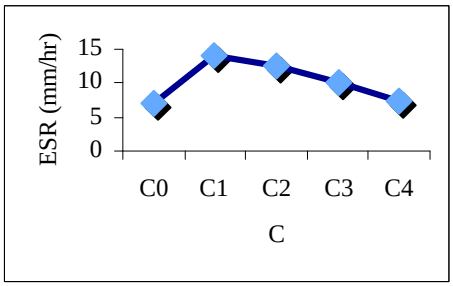


Fig. 5c.

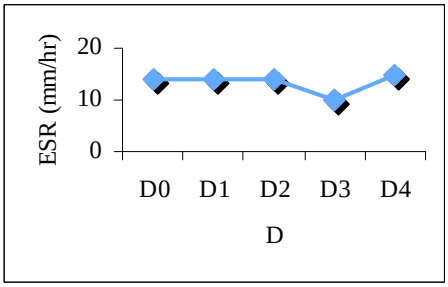


Fig. 5d.

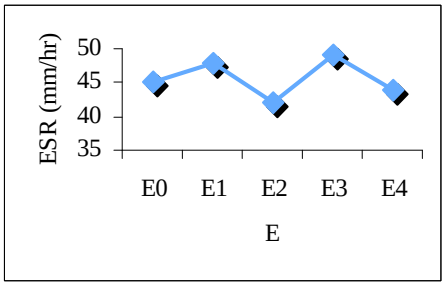


Fig. 5e.

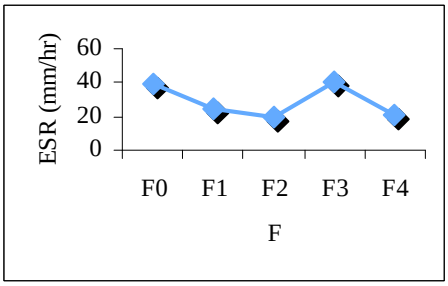


Fig. 5f

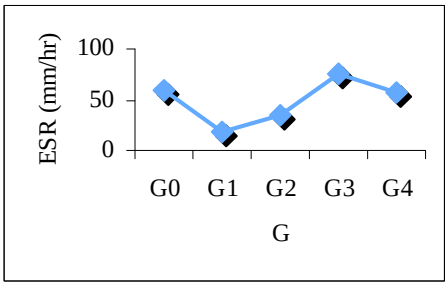


Fig. 5g

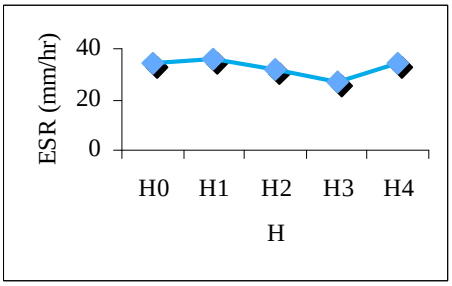


Fig. 5h

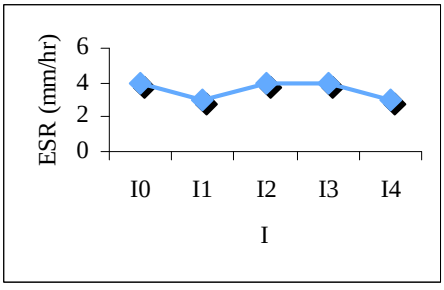


Fig. 5i

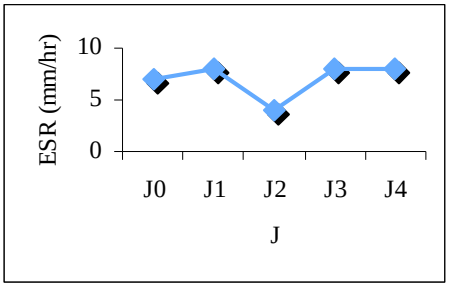


Fig. 5j

Appendix D (Continued)

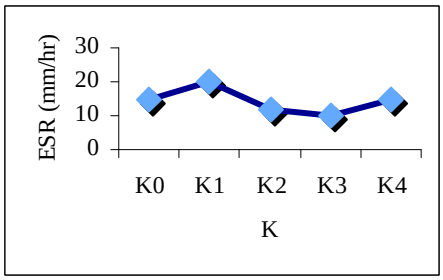


Fig. 5k

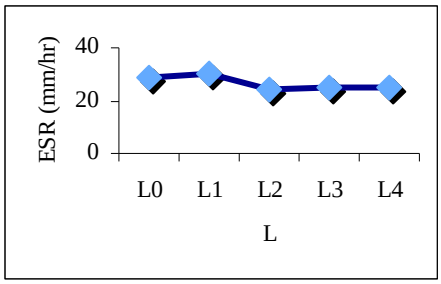


Fig. 5l

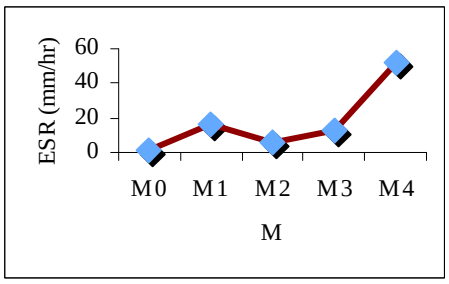


Fig. 5m

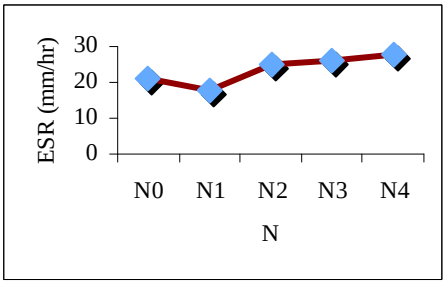


Fig. 5n

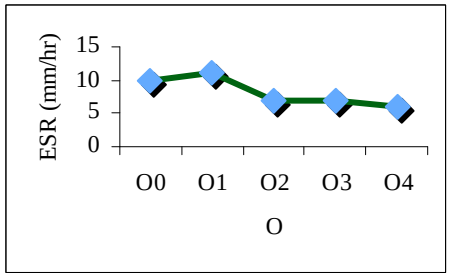


Fig. 5o

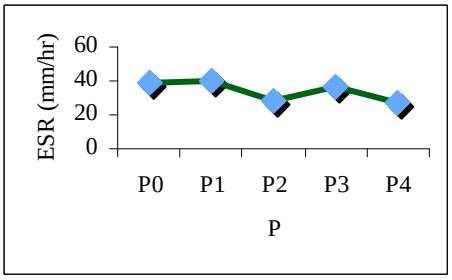


Fig. 5p

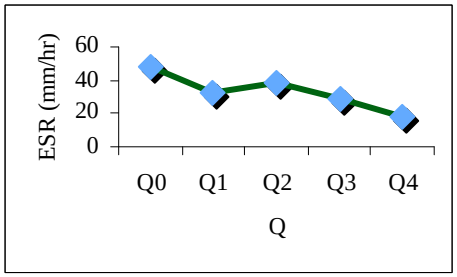


Fig. 5q

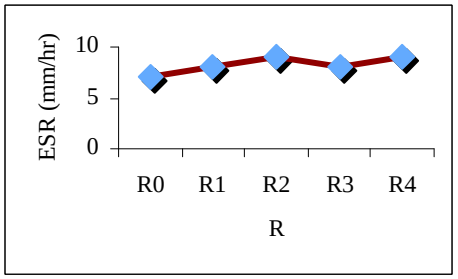


Fig. 5r

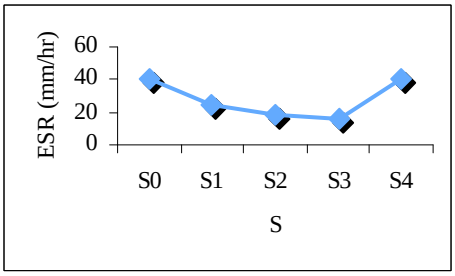


Fig. 5s

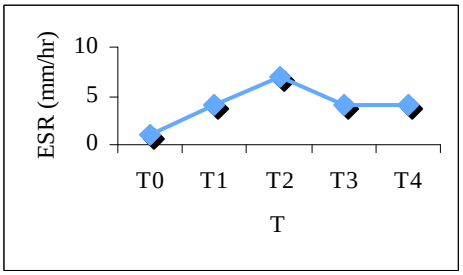


Fig. 5t

Appendix E

Figure 11. C-Reactive Protein Results

Note: In the following figures, the capital letter denotes the patient (from A to T), the 0-4 numbers represent the ozone concentration (4 being the highest ozone concentration). Green lines represent a positive effect, red lines an adverse effect.

For example: B2 means sample from patient B, 2nd highest ozone concentration treatment

Appendix E (Continued)

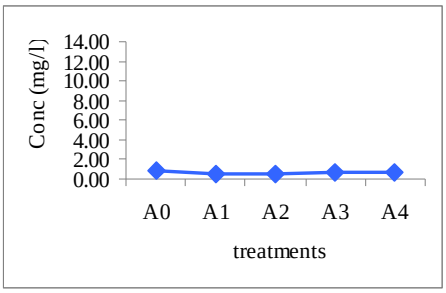


Fig. 11a

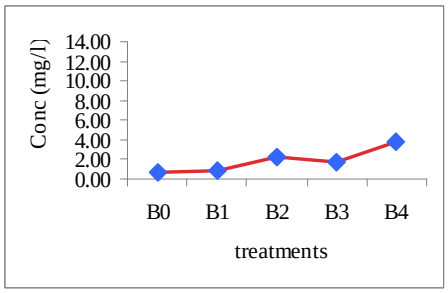


Fig. 11b

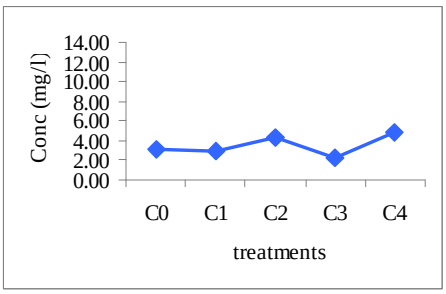


Fig. 11c

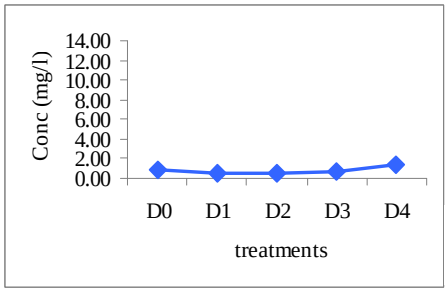


Fig. 11d

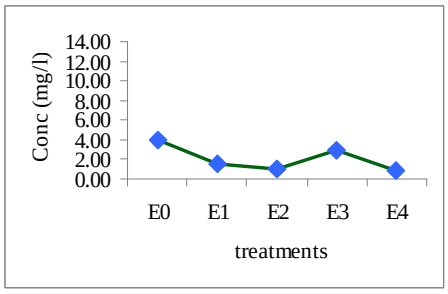


Fig. 11e

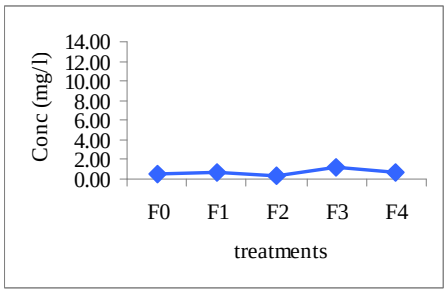


Fig. 11f

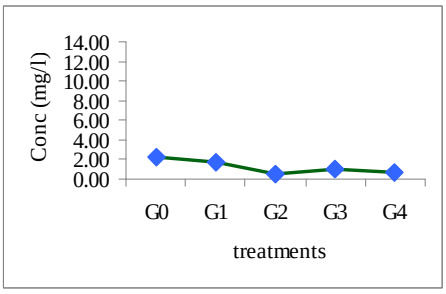


Fig. 11g

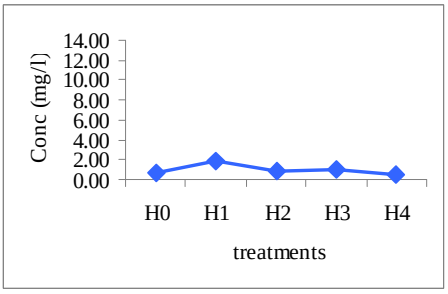


Fig. 11h

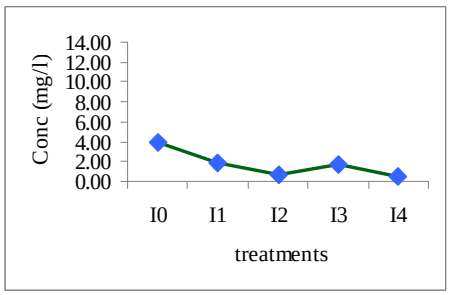


Fig. 11i

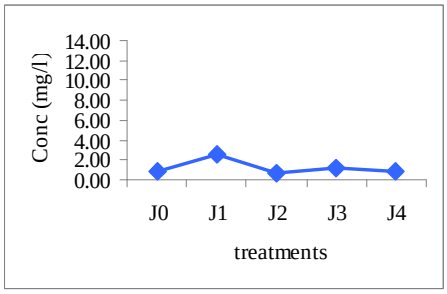


Fig. 11j

Appendix E (Continued)

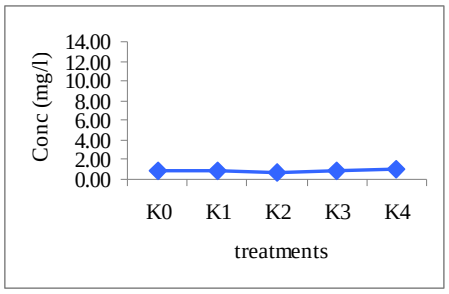


Fig. 11k

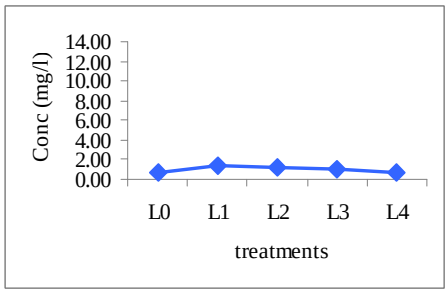


Fig. 11l

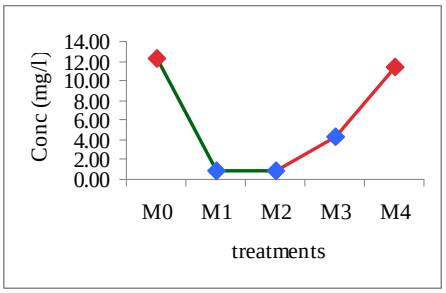


Fig. 11m

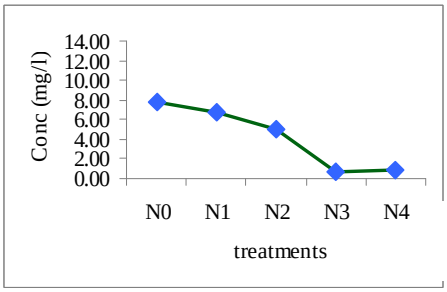


Fig. 11n

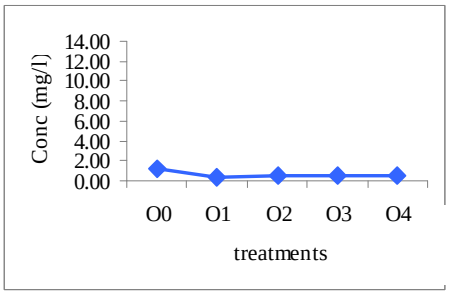


Fig. 11o

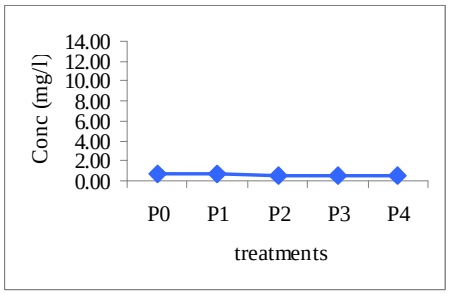


Fig. 11p

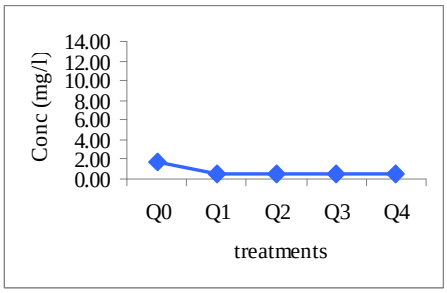


Fig. 11q

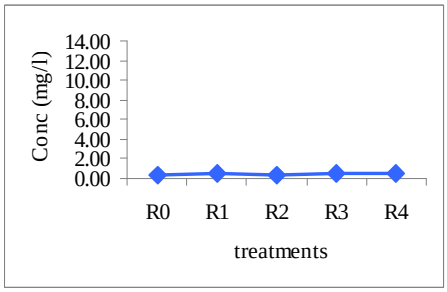


Fig. 11r

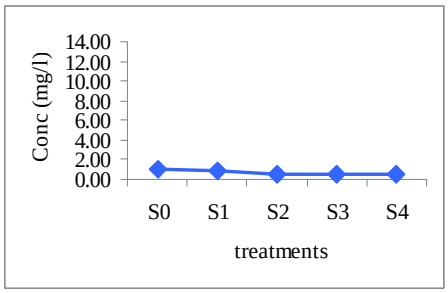


Fig. 11s

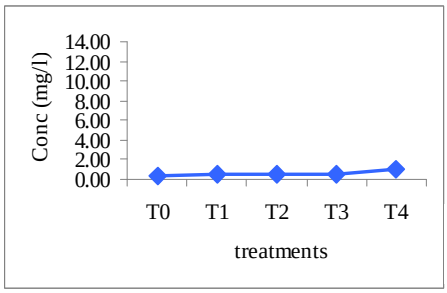


Fig. 11t

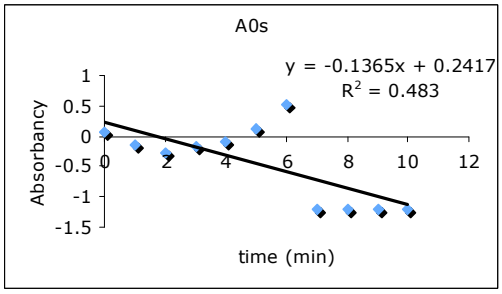
Appendix F

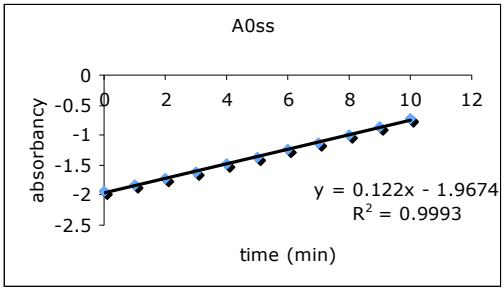
Figure 13. Glutathione Absorbancies for Ozone Treatments

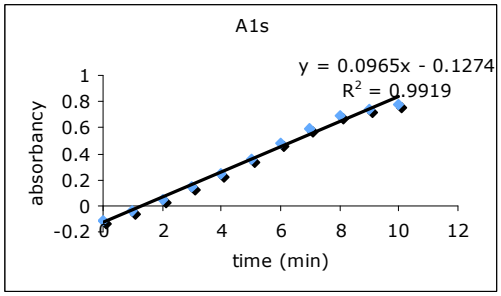
Note: In the following figures, the capital letter denotes the patient (from A to T), the 0-4 numbers represent the ozone concentration (4 being the highest ozone concentration), the small letter ‘s’ denotes a GSH sample and the double letter ‘ss’ denotes a GSSG sample.

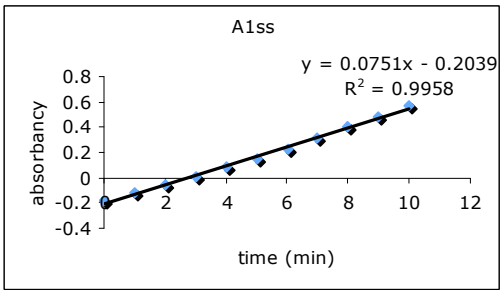
For example: A2s means patient A, 2nd highest ozone concentration treatment, GSH sample.

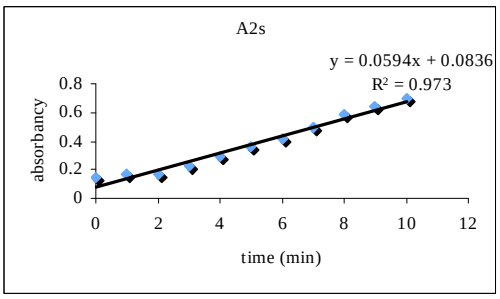
Appendix F (Continued)

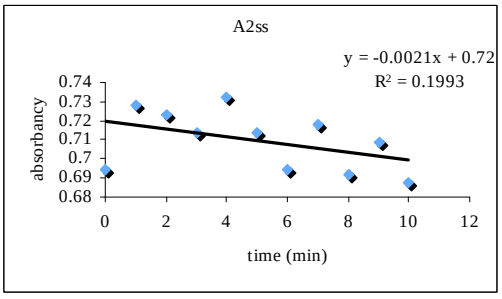


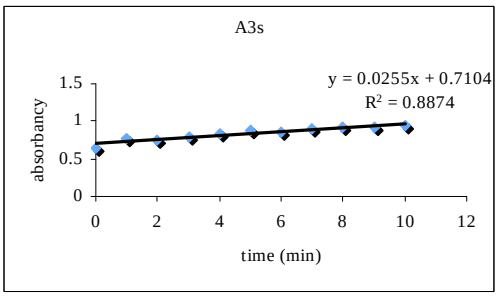


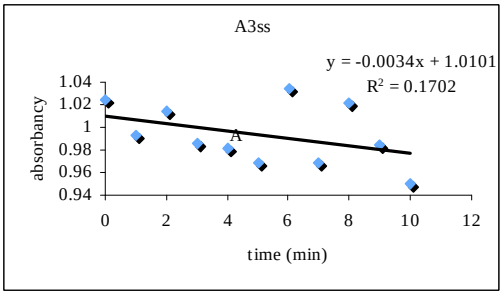


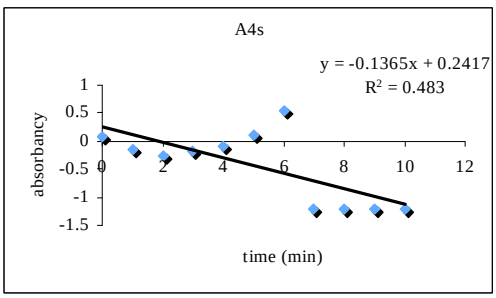


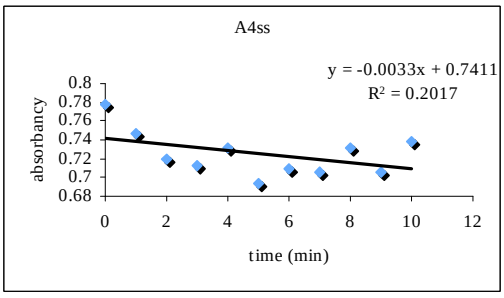






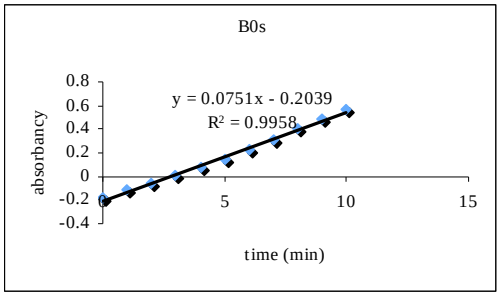


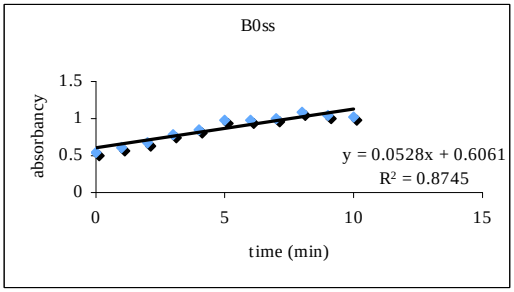


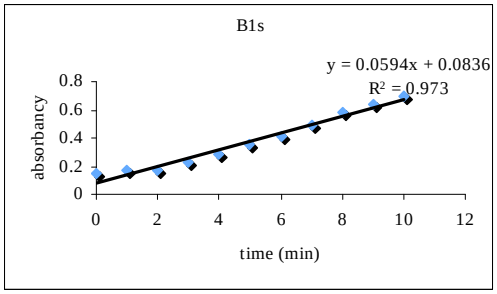


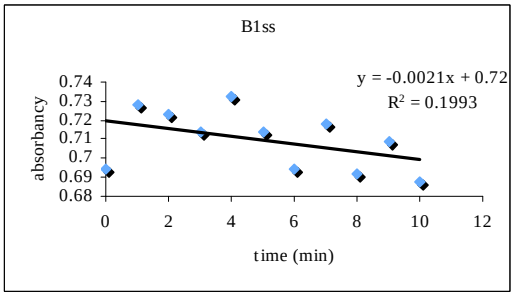
a. Glutathione absorbencies for patient A for the five treatments

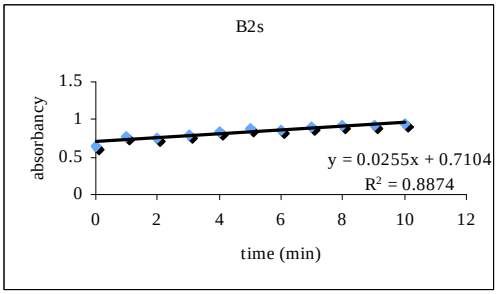
Appendix F (Continued)

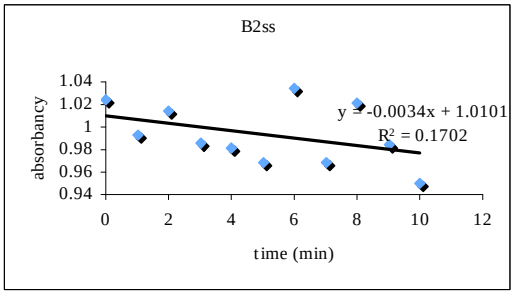


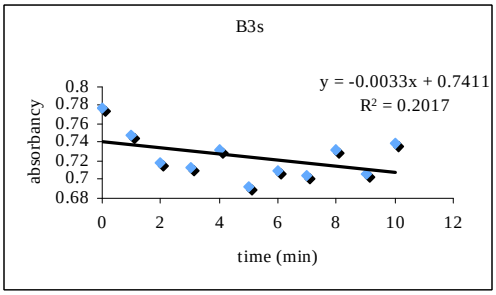


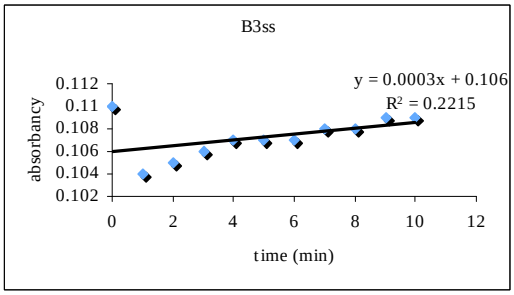


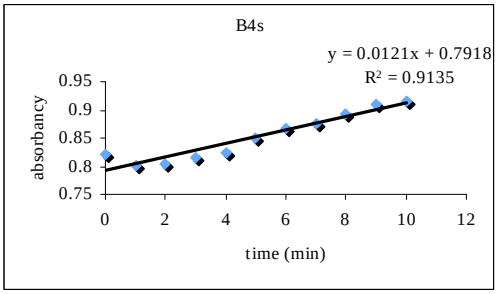


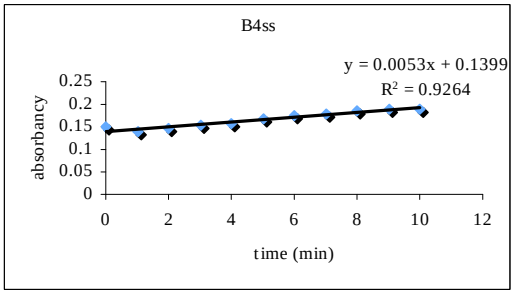






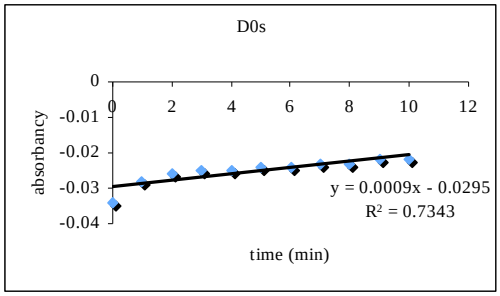


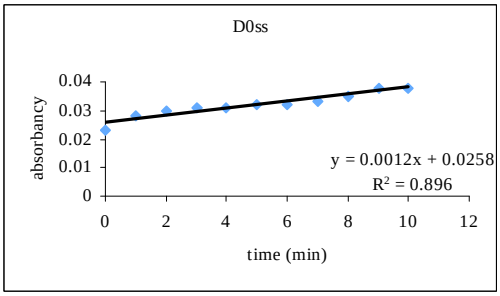


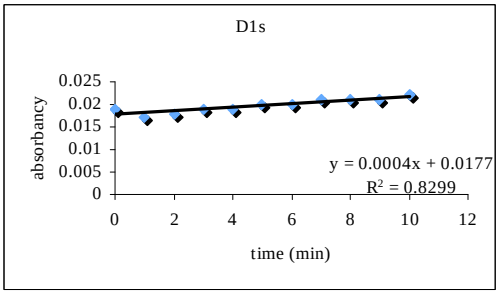


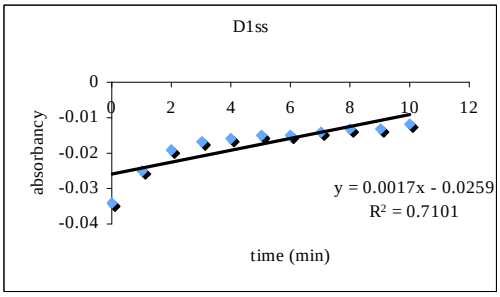
b. Glutathione absorbencies for patient B for the five treatments

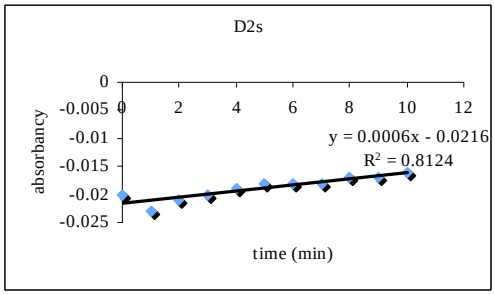
Appendix F (Continued)

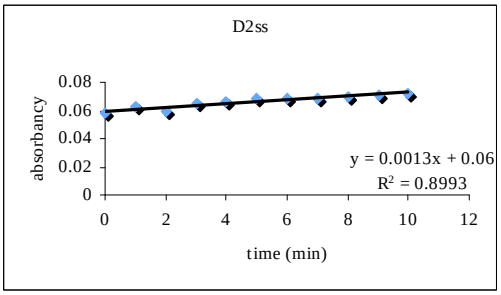


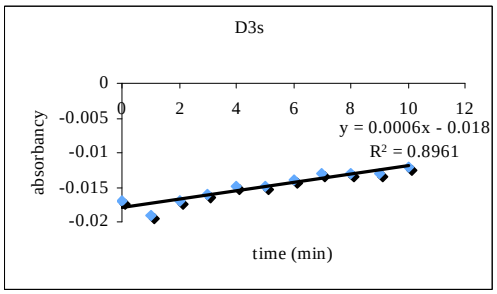


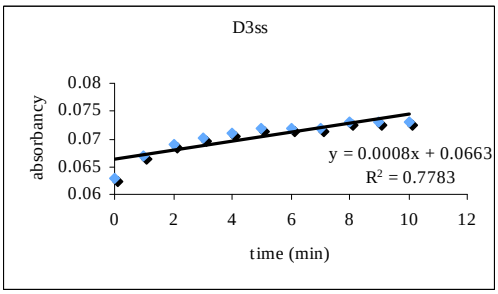


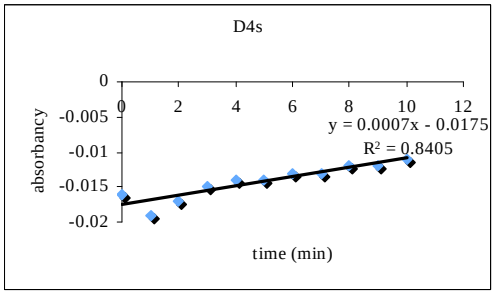


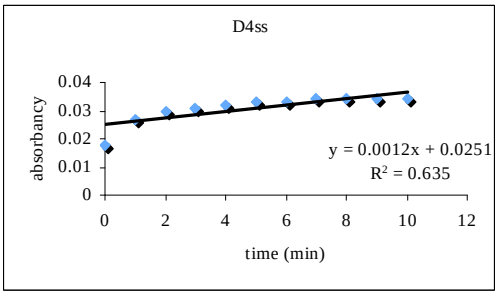






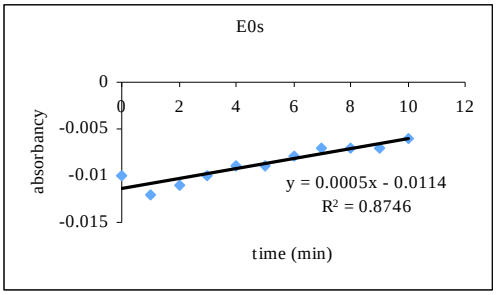


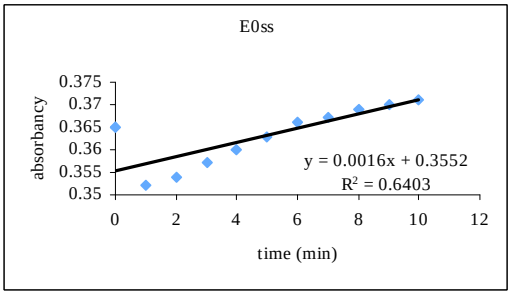


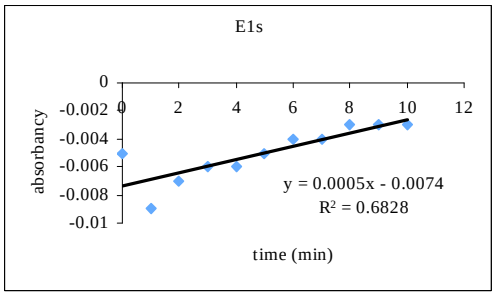


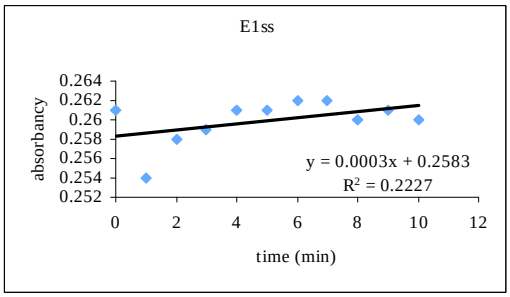
d. Glutathione absorbencies for patient D for the five treatments

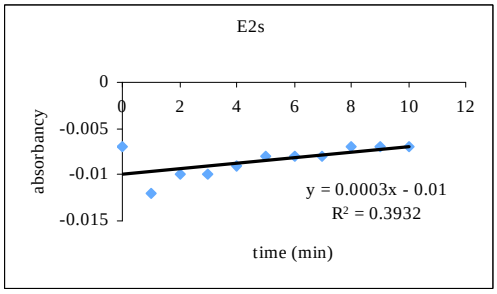
Appendix F (Continued)

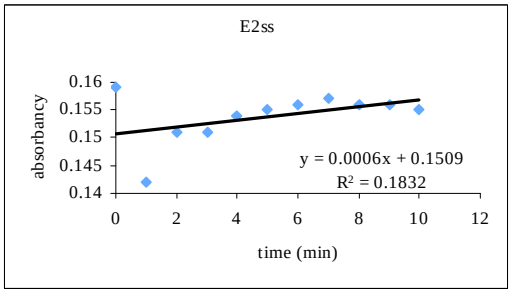


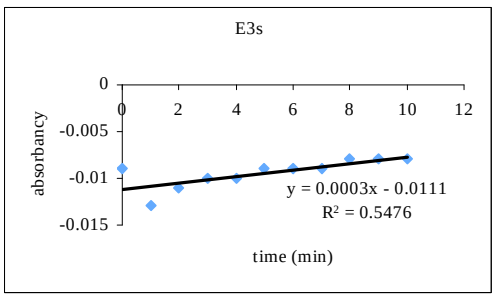


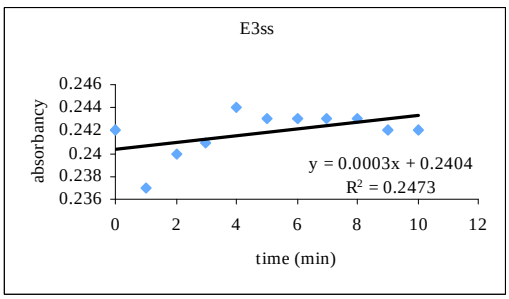


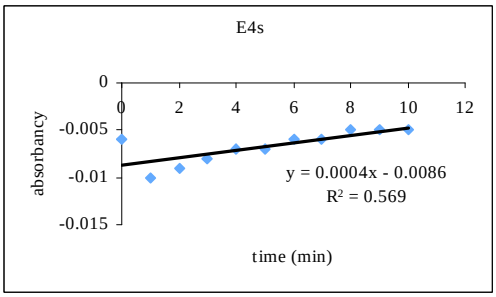


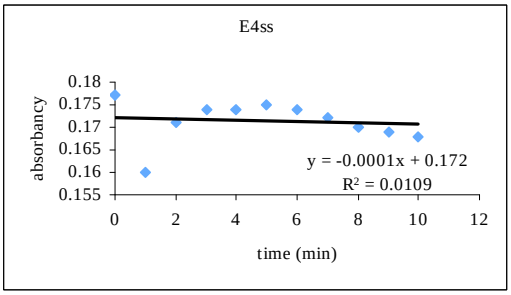






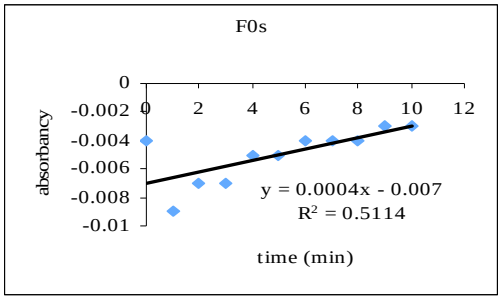


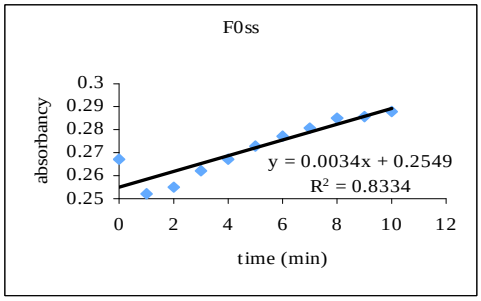


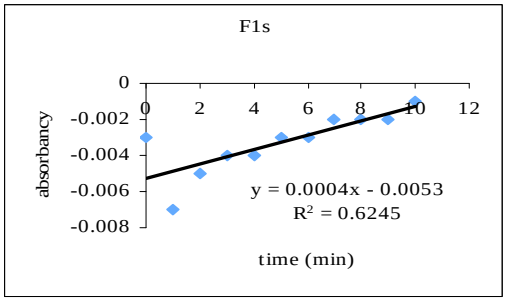


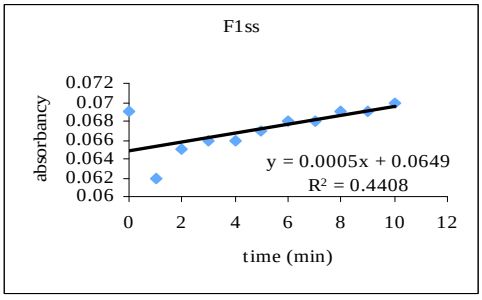
e. Glutathione absorbencies for patient E for the five treatments

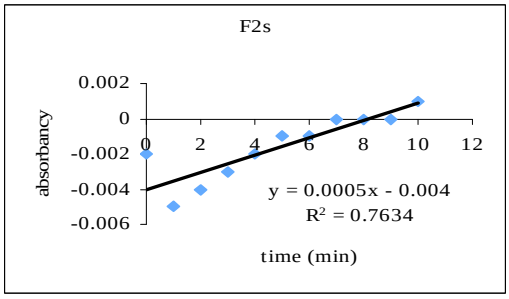
Appendix F (Continued)

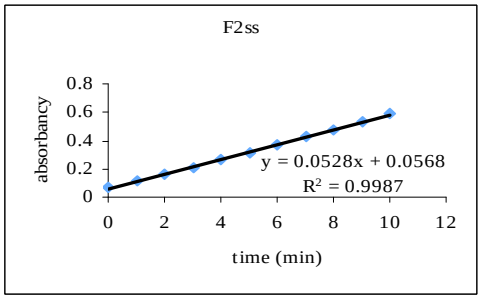


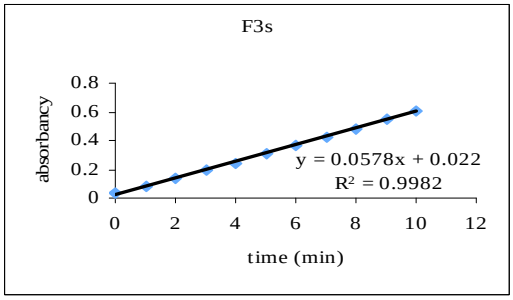


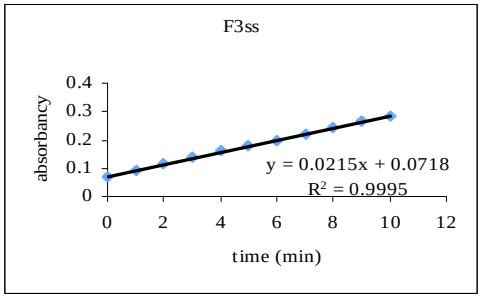


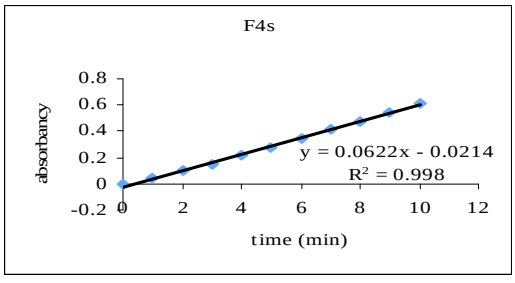


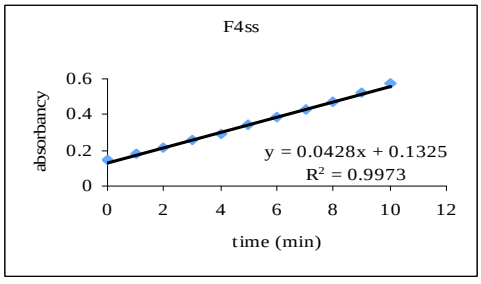






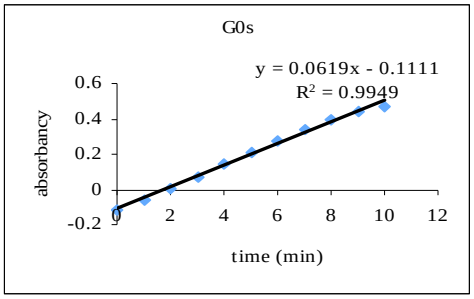


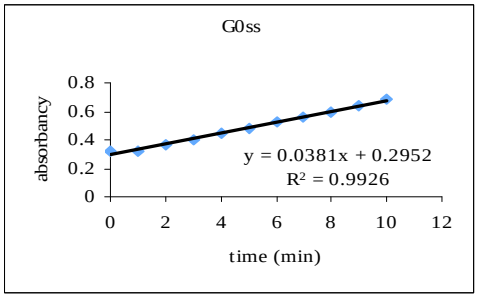


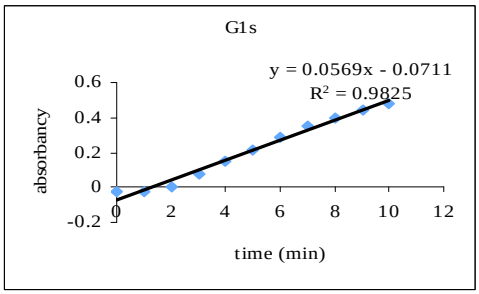


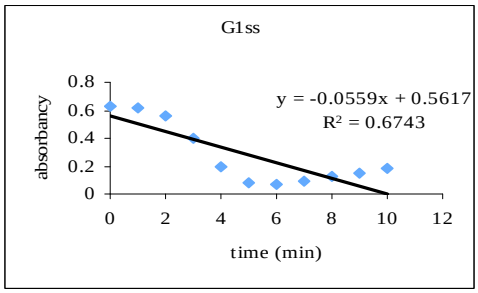
f. Glutathione absorbencies for patient F for the five treatments

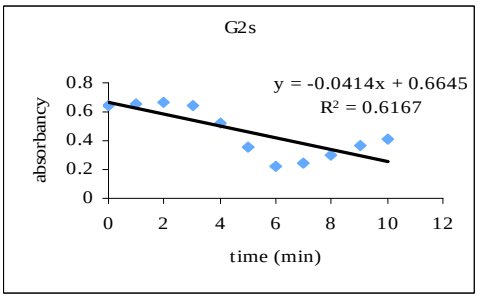
Appendix F (Continued)

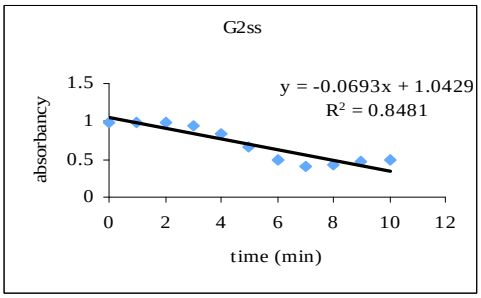


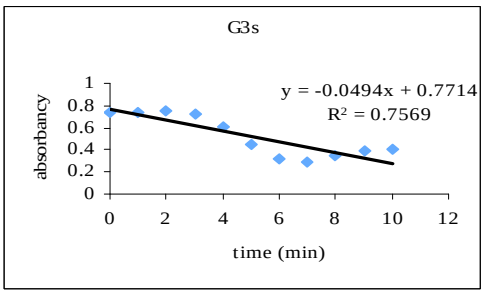


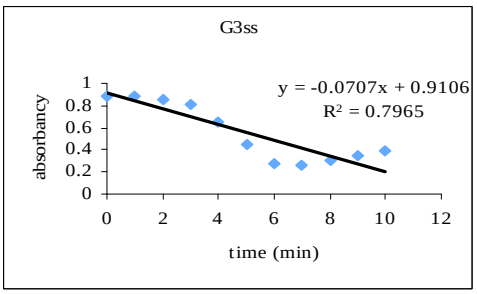


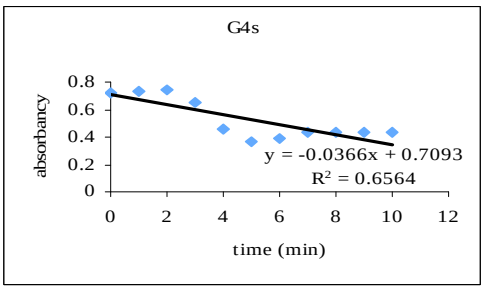


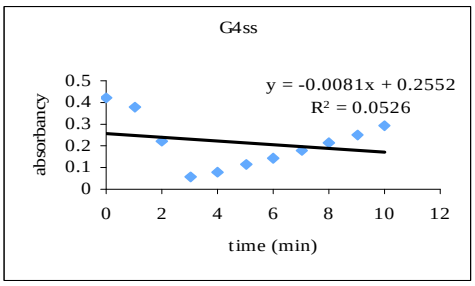






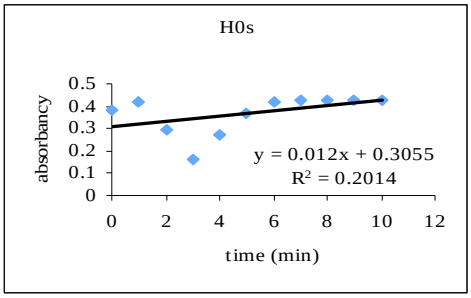


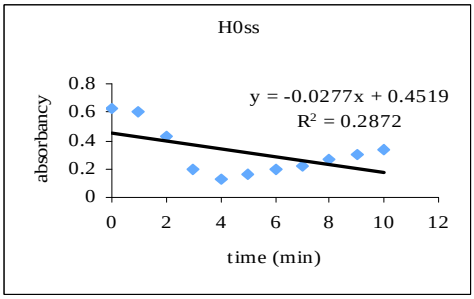


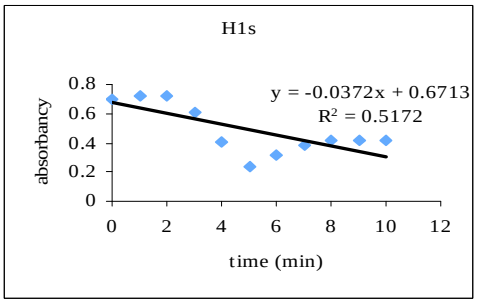


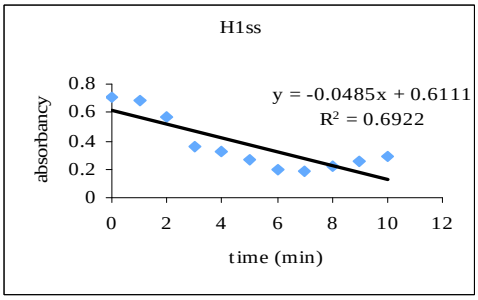
g. Glutathione absorbencies for patient G for the five treatments

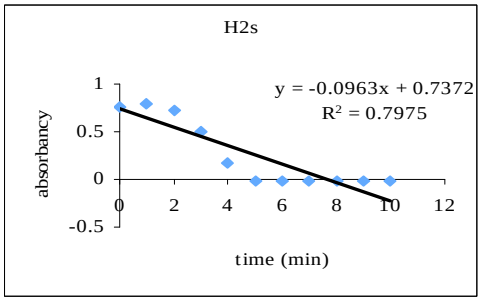
Appendix F (Continued)

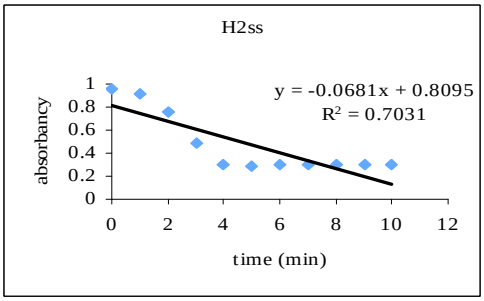


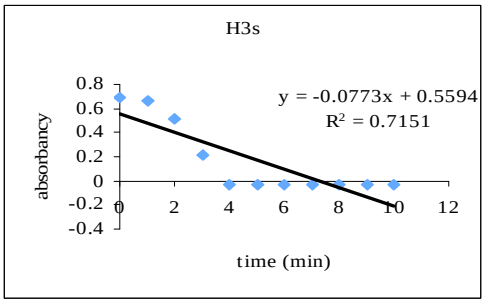


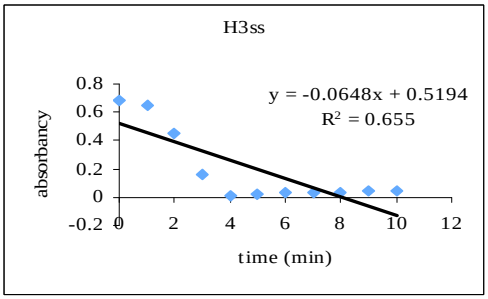


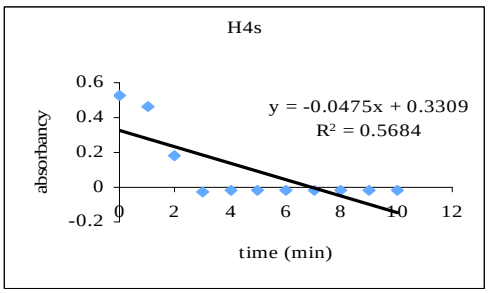


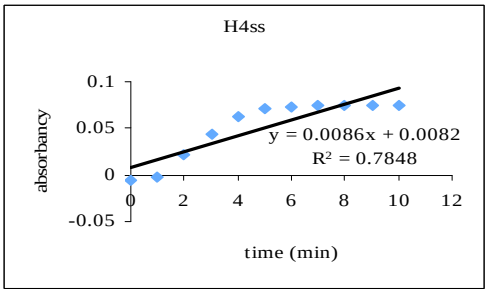






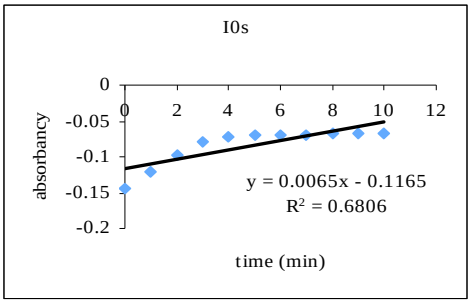


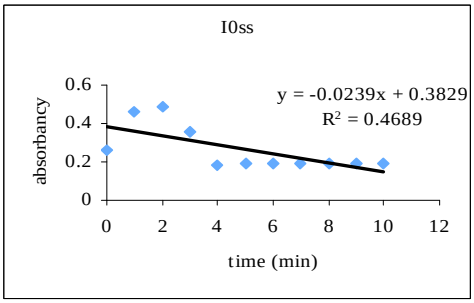


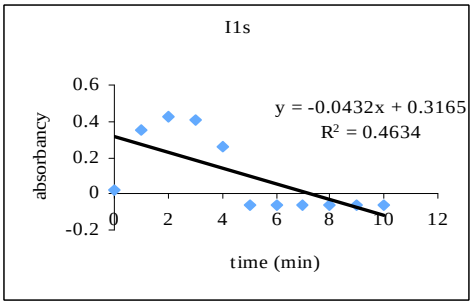


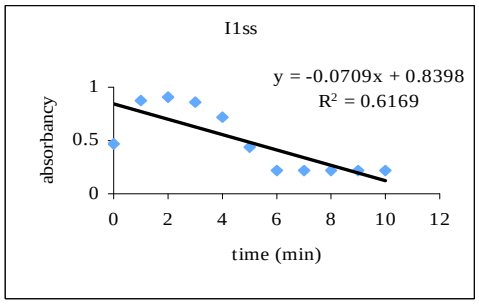
h. Glutathione absorbencies for patient H for the five treatments

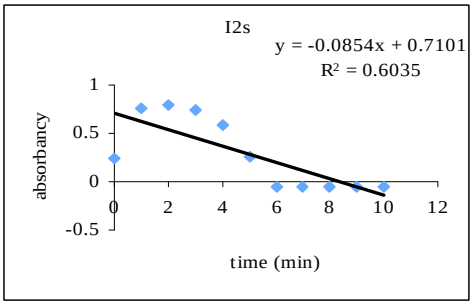
Appendix F (Continued)

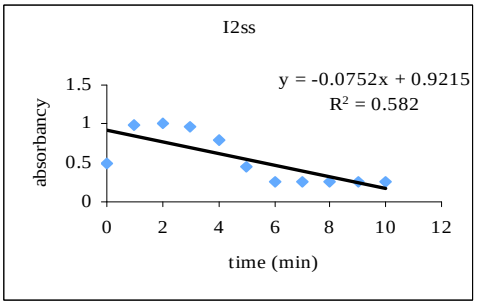


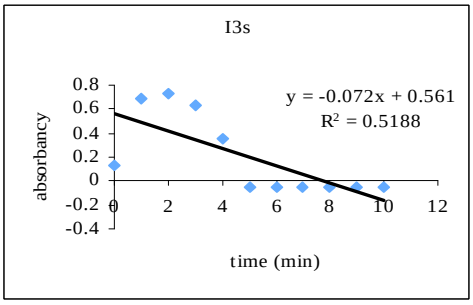


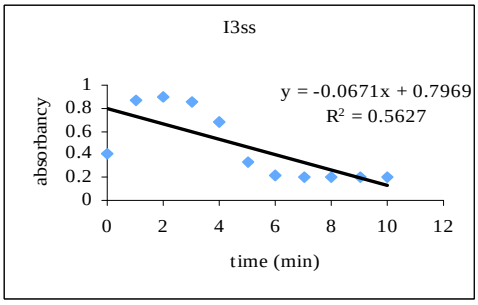


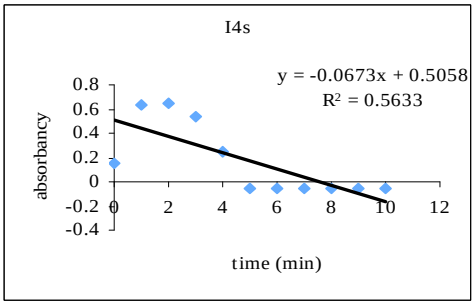


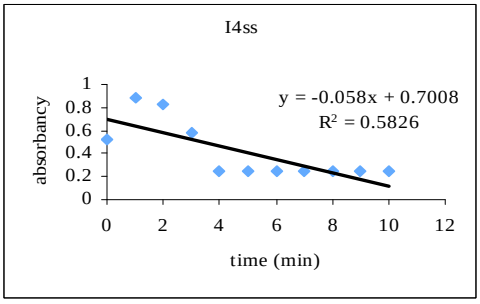






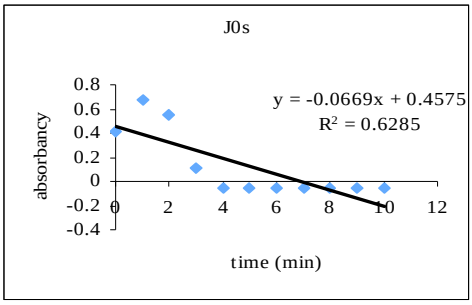


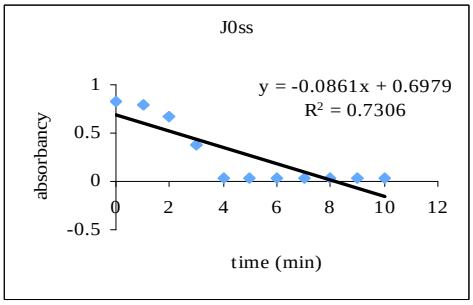


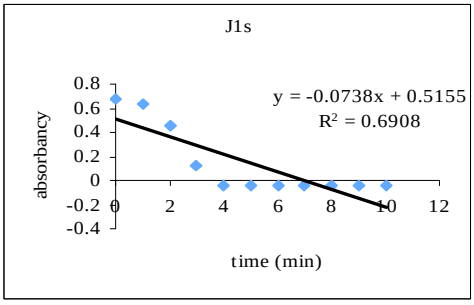


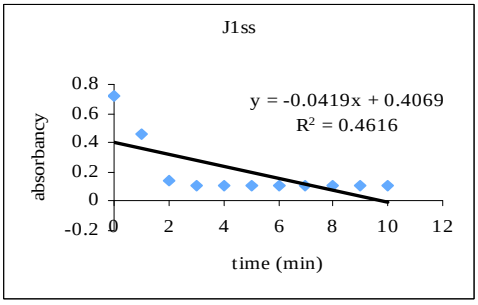
i. Glutathione absorbencies for patient I for the five treatments

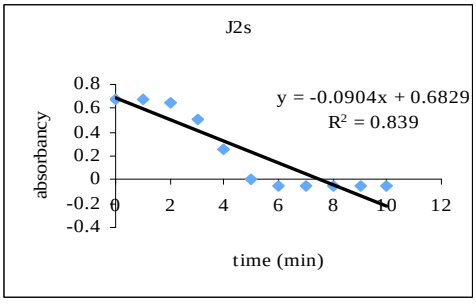
Appendix F (Continued)

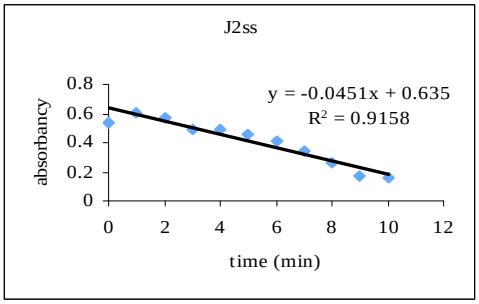


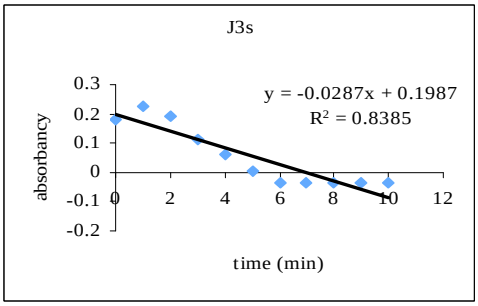


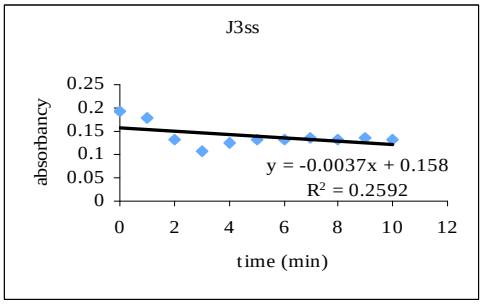


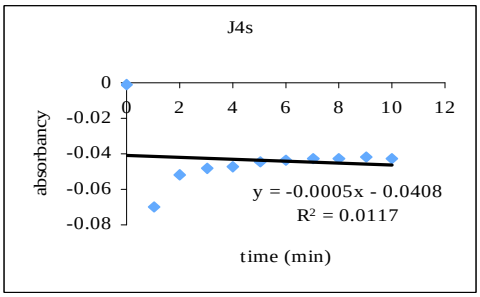


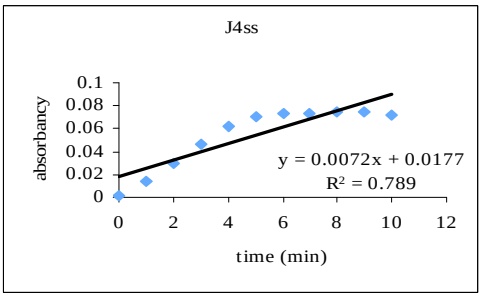






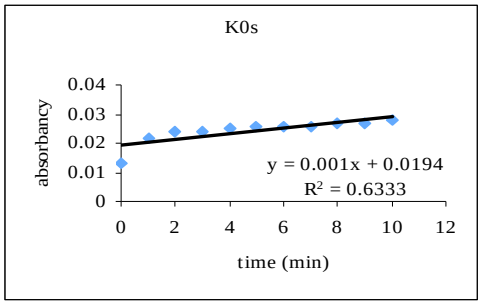


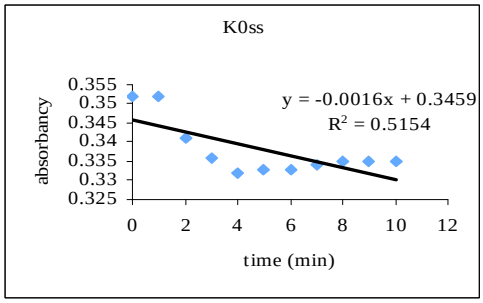


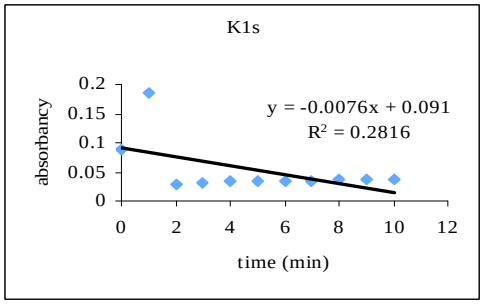


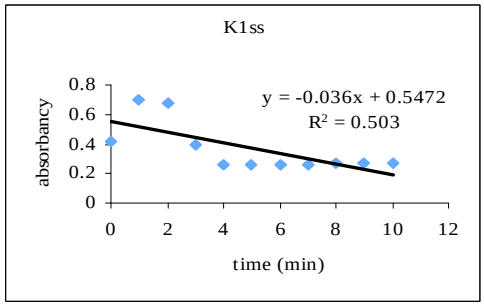
j. Glutathione absorbencies for patient J for the five treatments

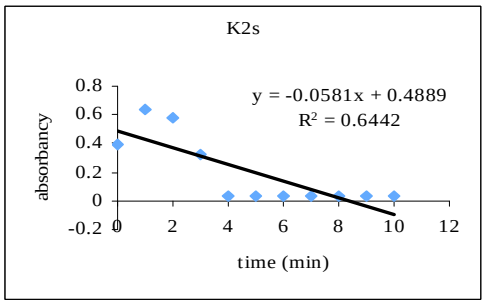
Appendix F (Continued)

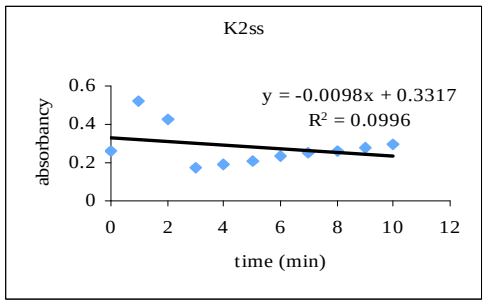


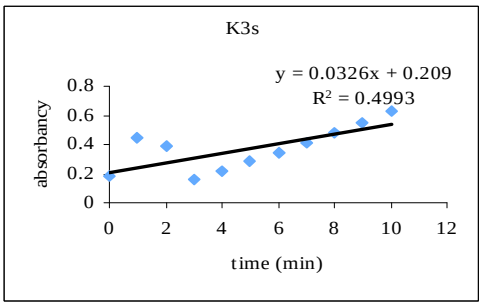


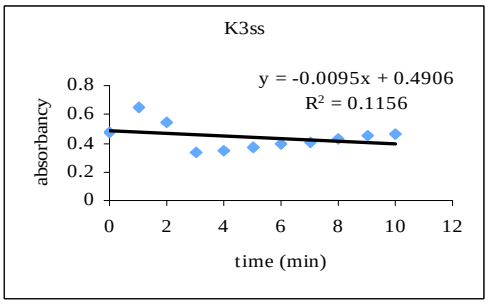


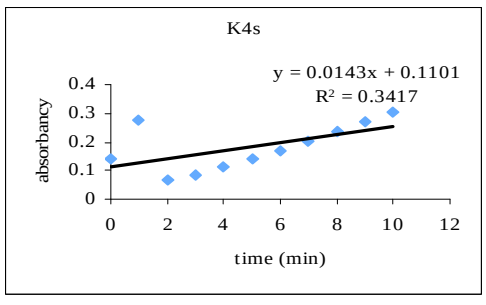


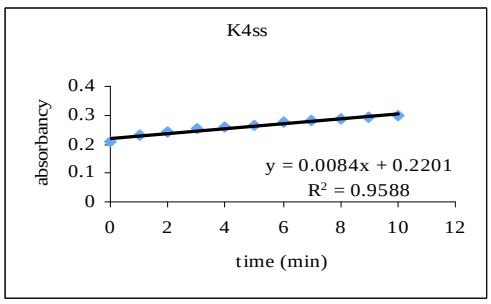






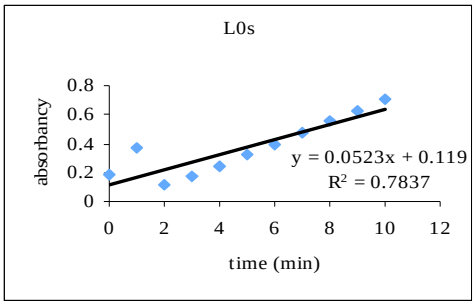


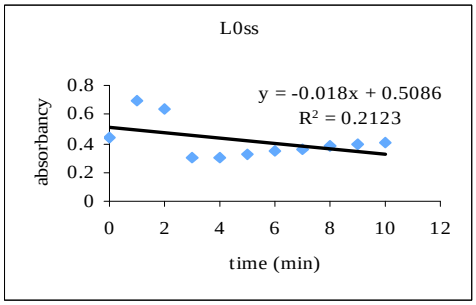


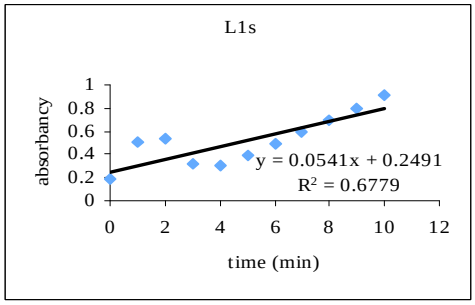


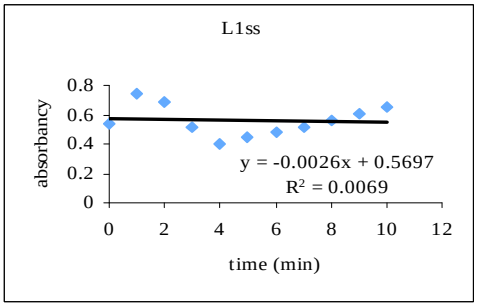
k. Glutathione absorbencies for patient K for the five treatments

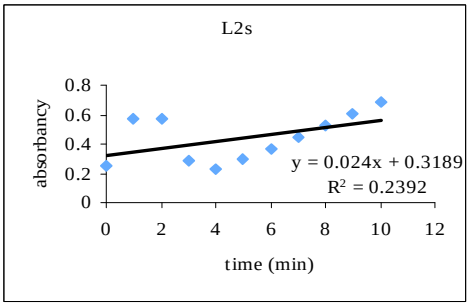
Appendix F (Continued)

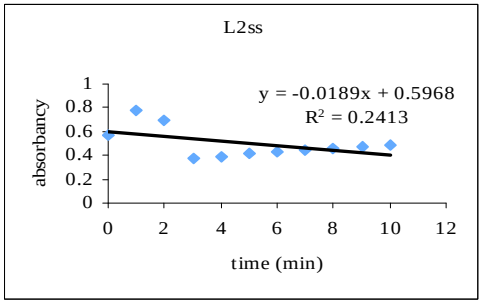


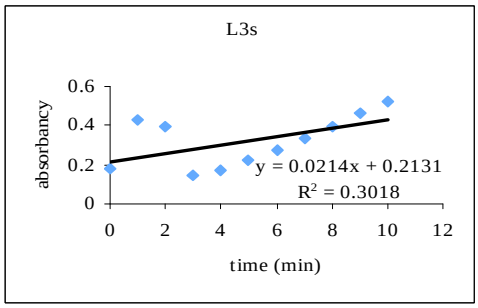


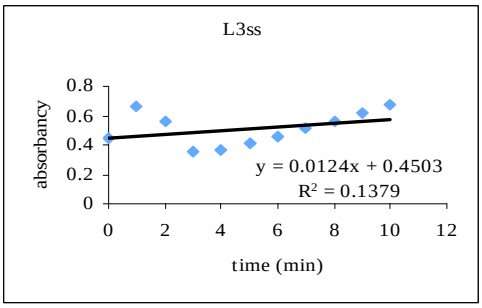


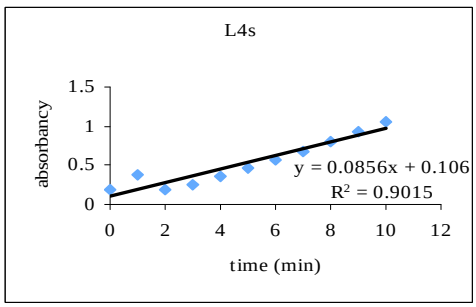


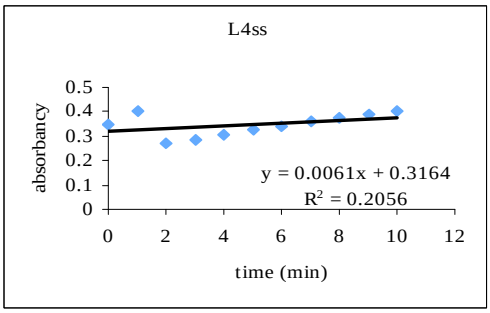






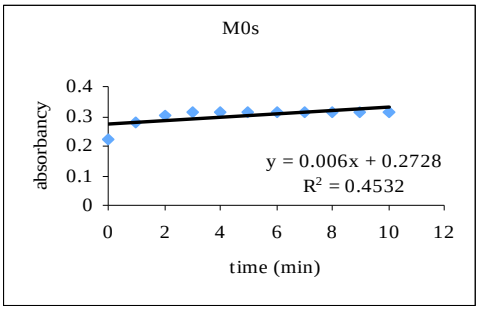


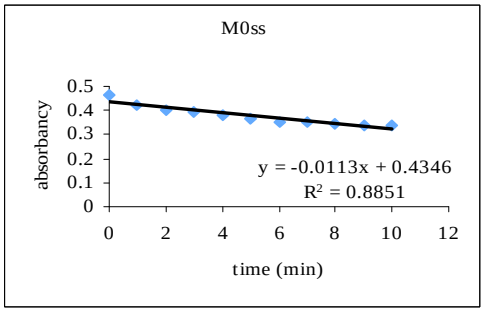


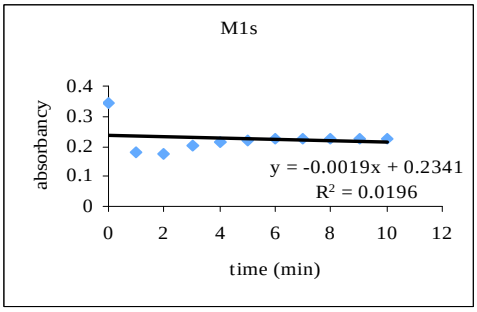


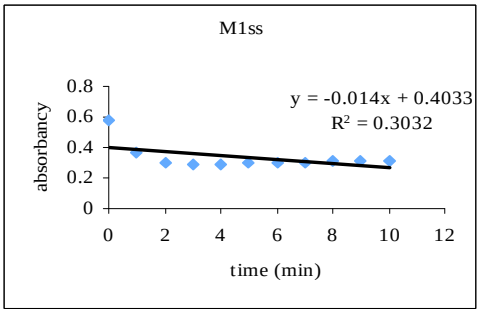
l. Glutathione absorbencies for patient L for the five treatments

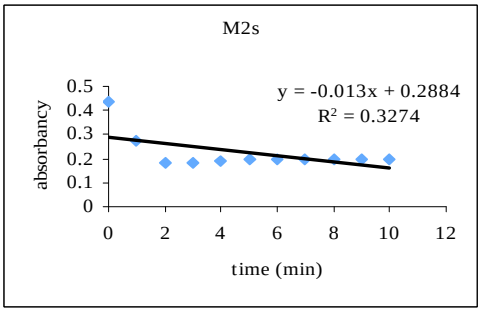
Appendix F (Continued)

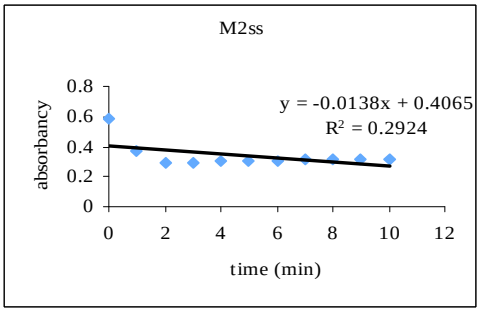


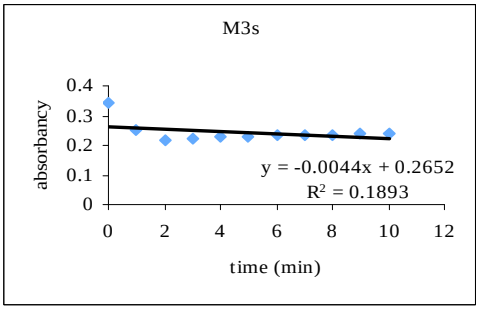


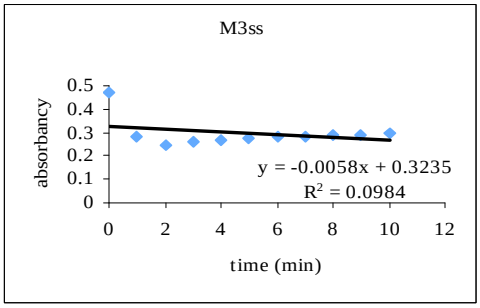


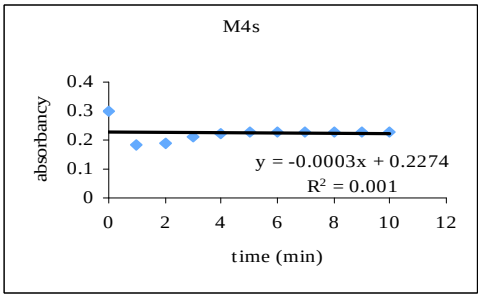


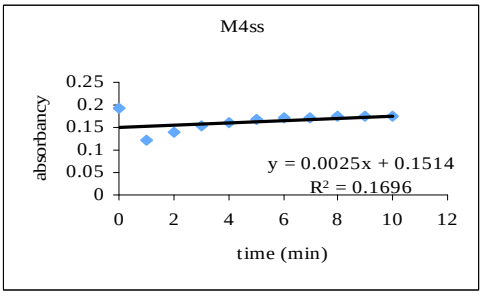






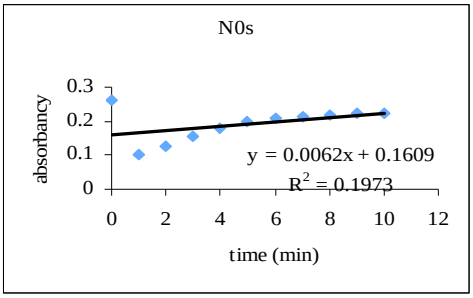


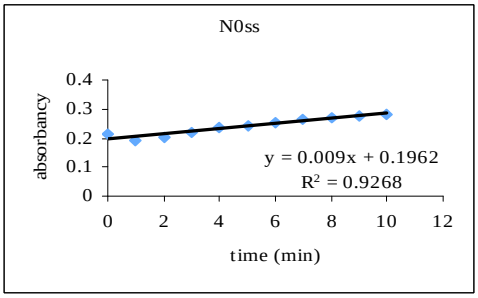


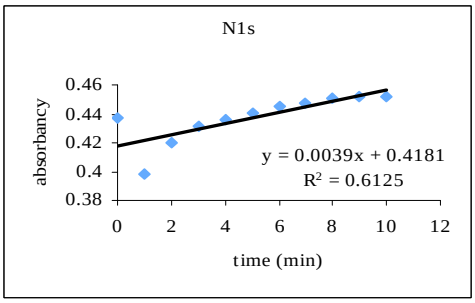


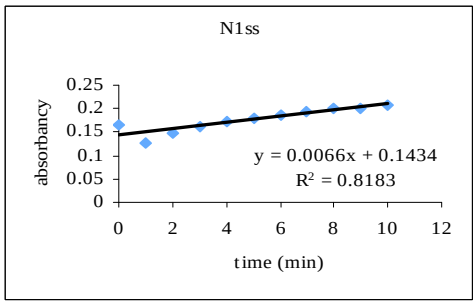
m. Glutathione absorbencies for patient M for the five treatments

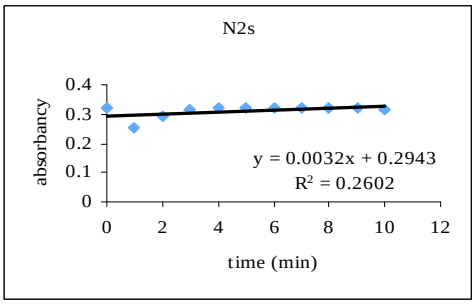
Appendix F (Continued)

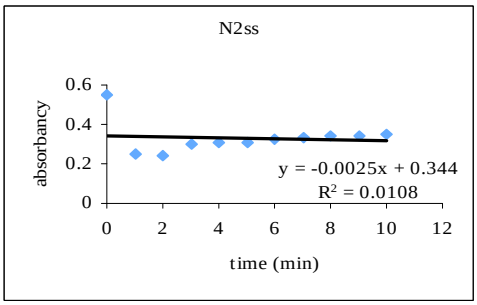


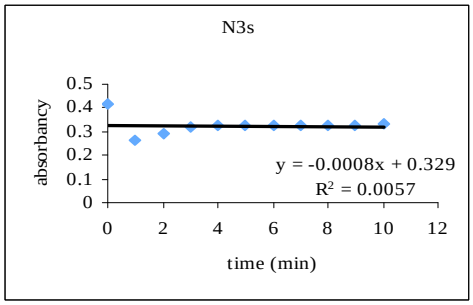


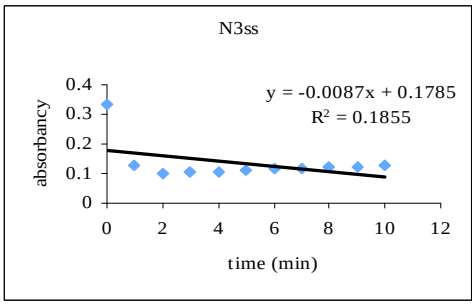


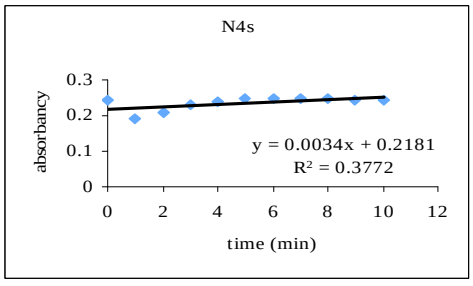


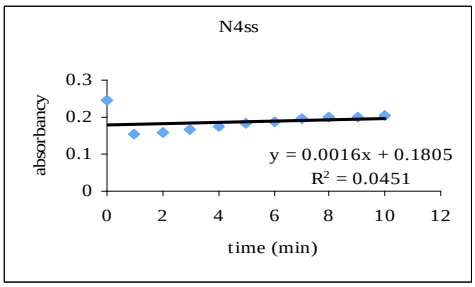






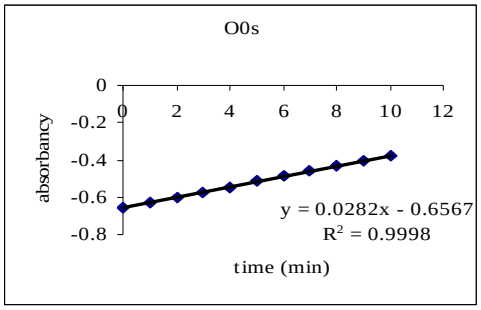


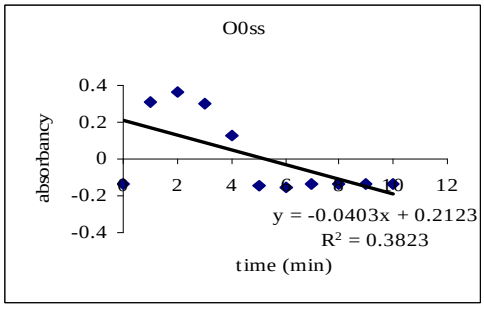


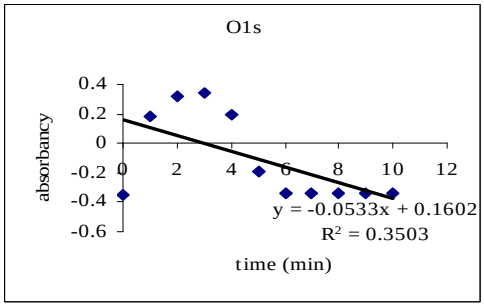


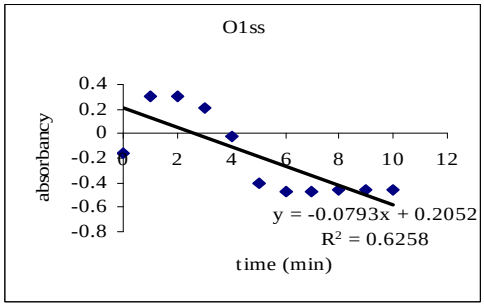
n. Glutathione absorbencies for patient N for the five treatments

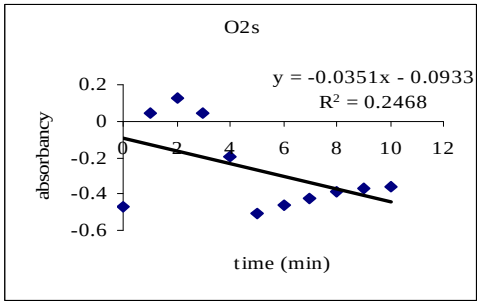
Appendix F (Continued)

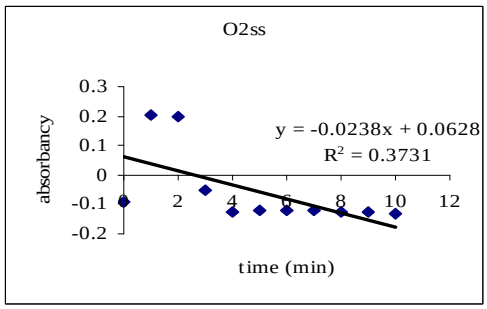


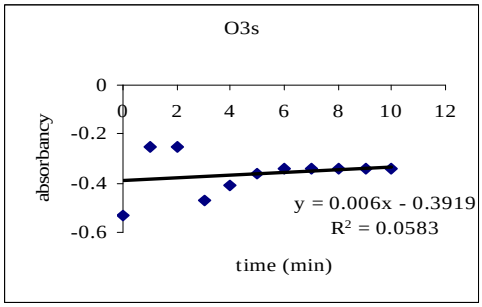


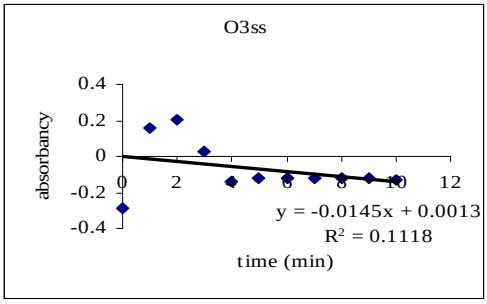


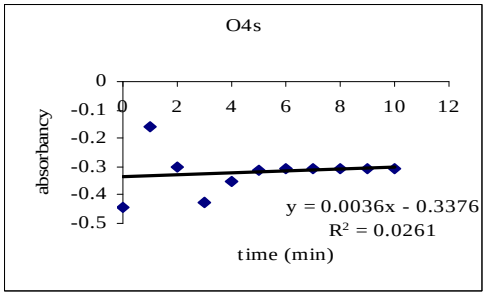


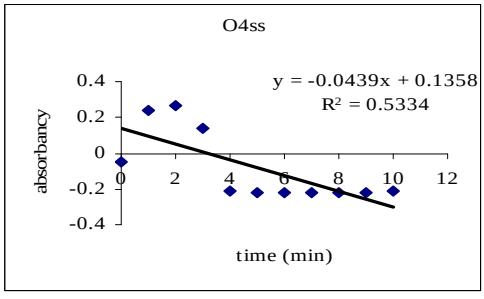






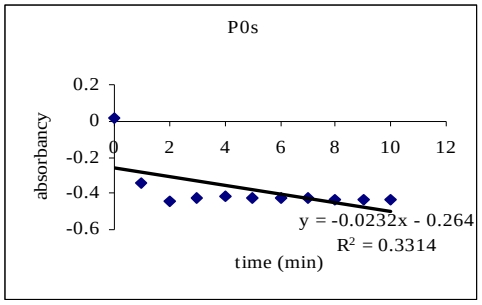


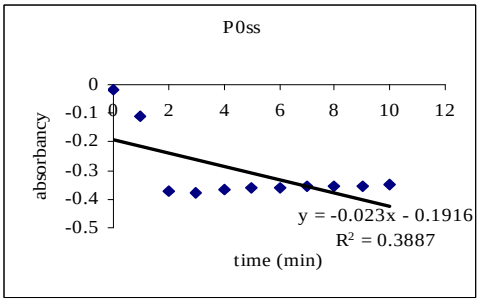


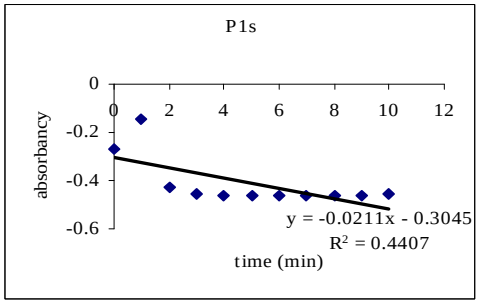


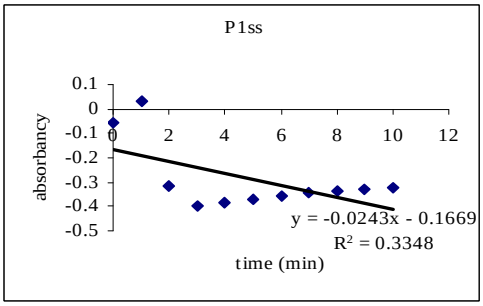
o. Glutathione absorbencies for patient O for the five treatments

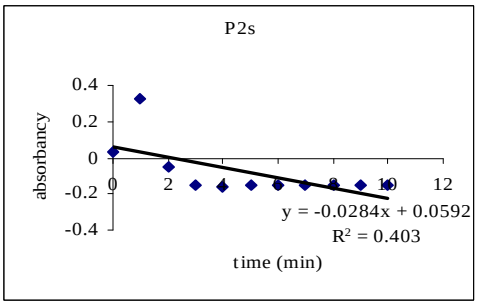
Appendix F (Continued)

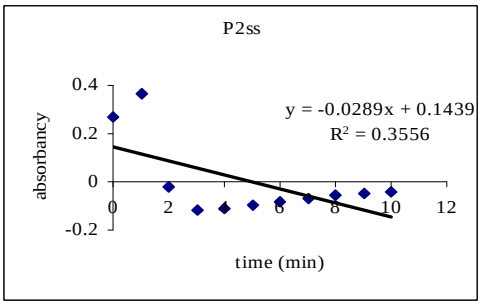


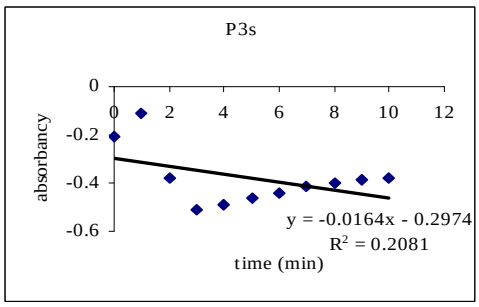


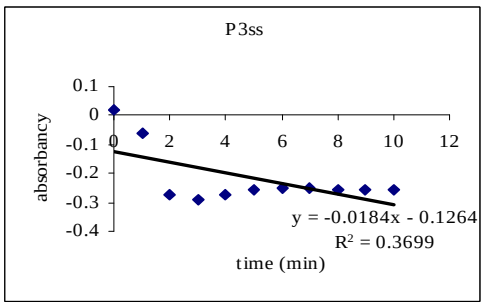


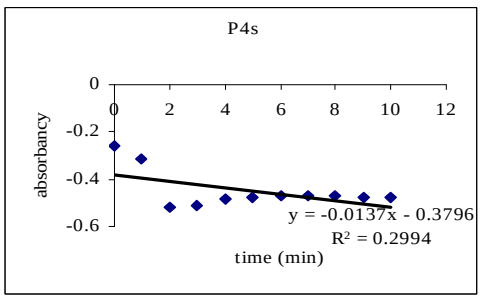


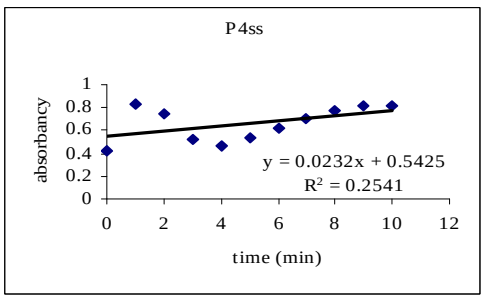






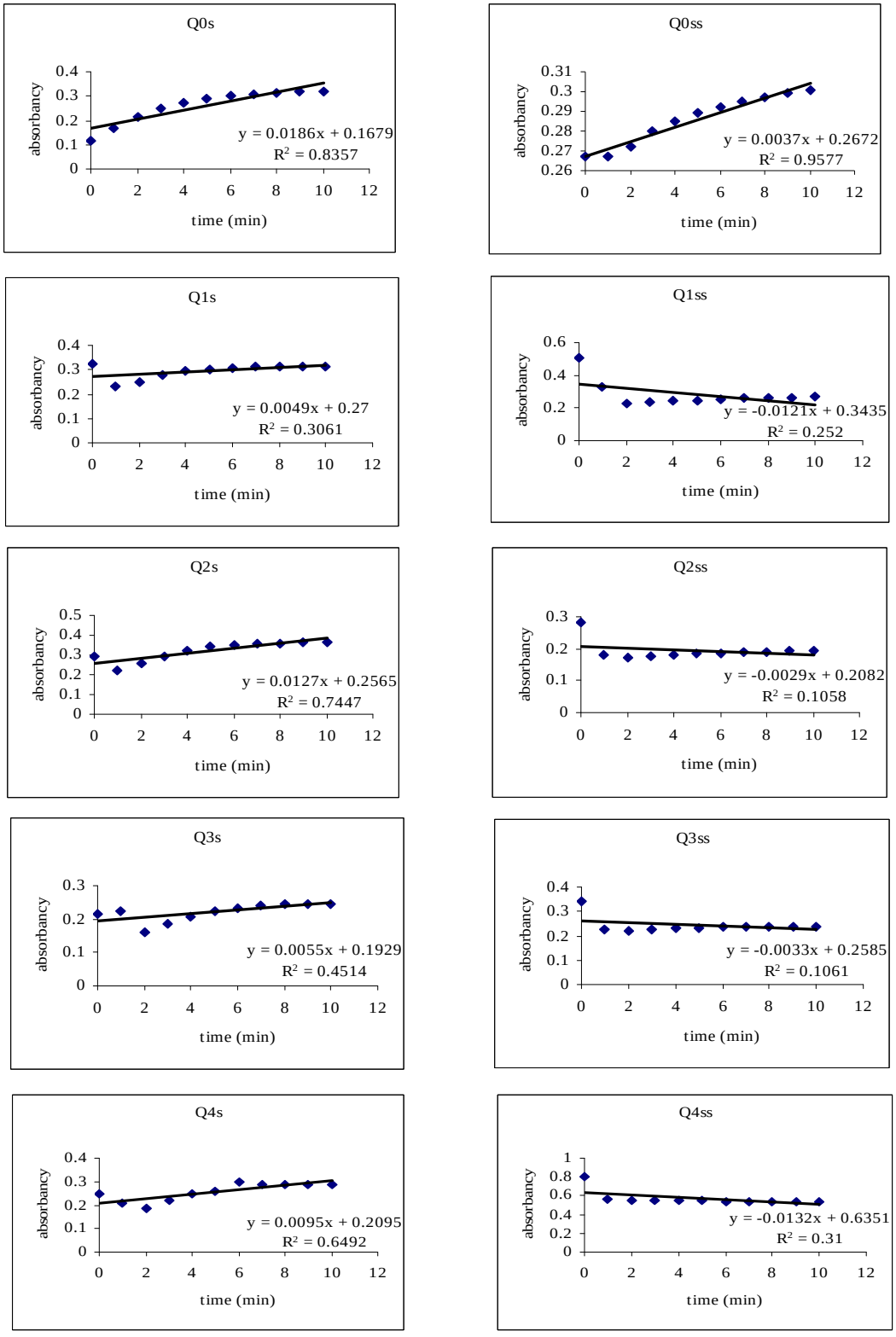






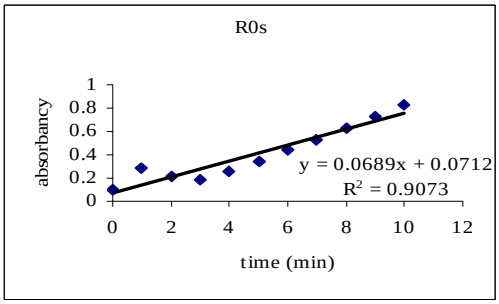
p. Glutathione absorbencies for patient P for the five treatments

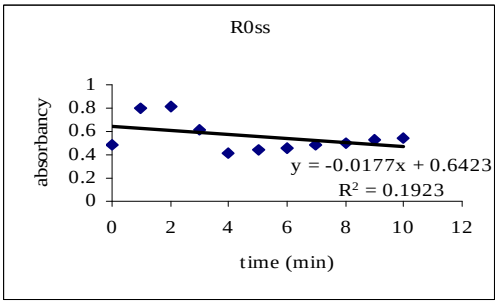
Appendix F (Continued)

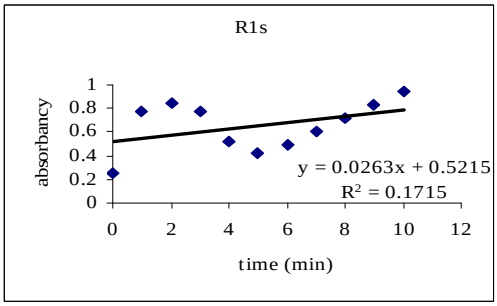


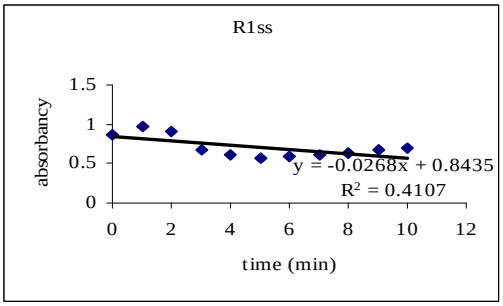
q. Glutathione absorbencies for patient Q for the five treatments

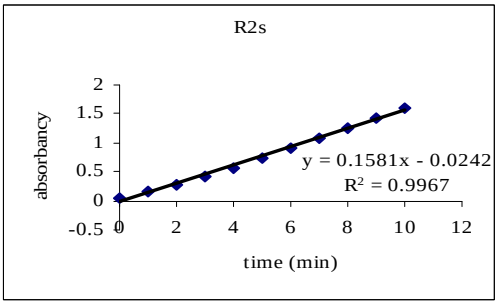
Appendix F (Continued)

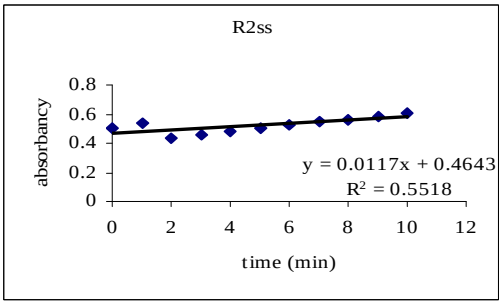


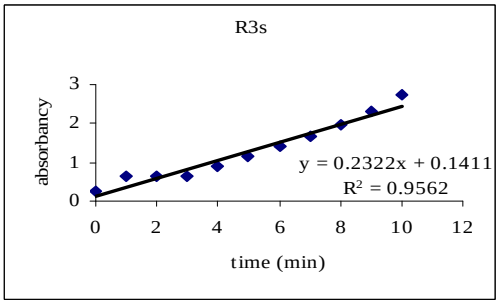


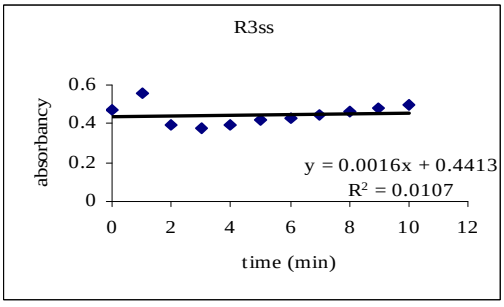


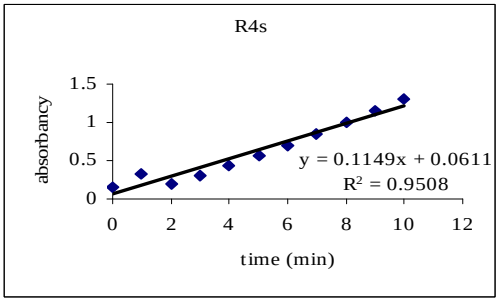


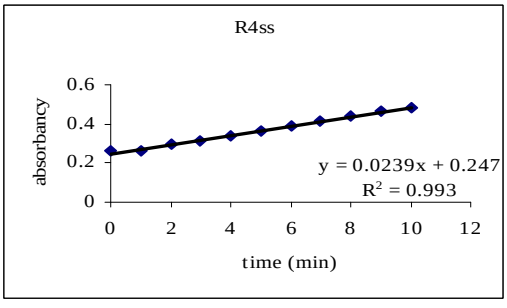






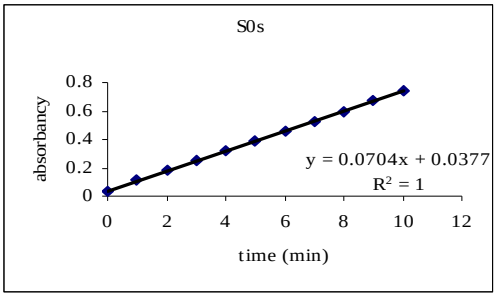


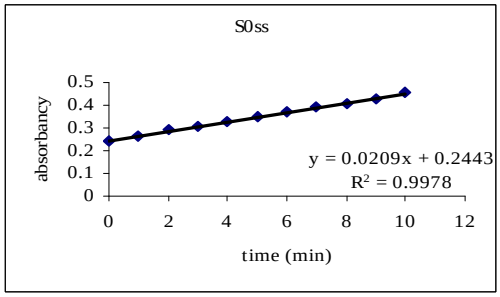


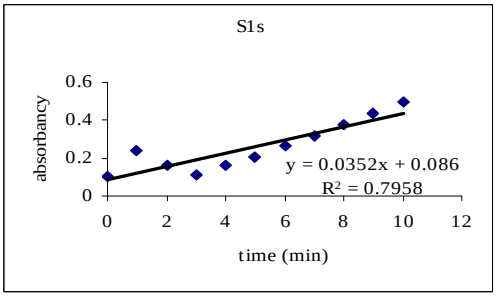


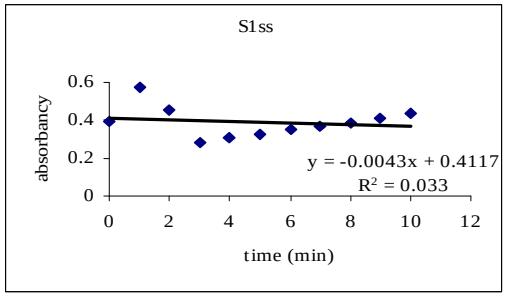
r. Glutathione absorbencies for patient R for the five treatments

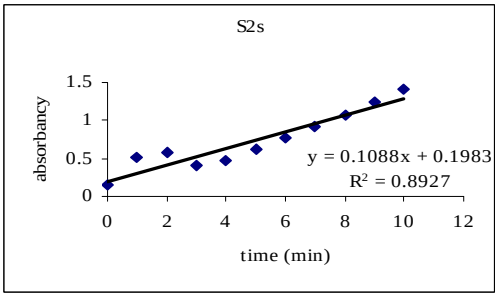
Appendix F (Continued)

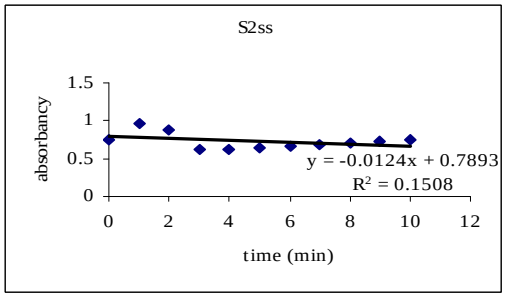


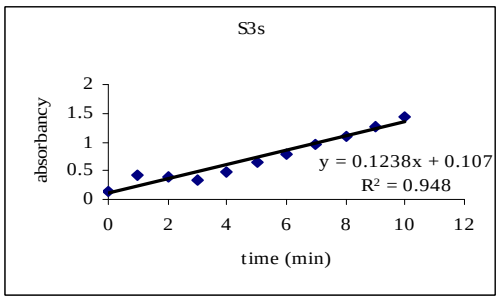


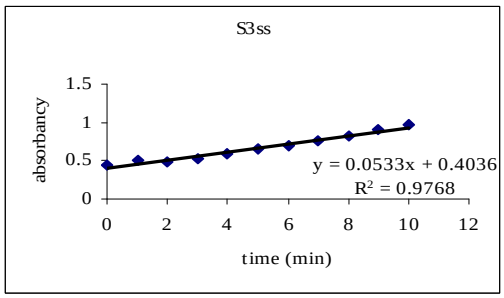


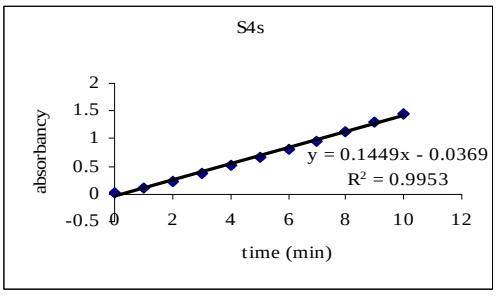


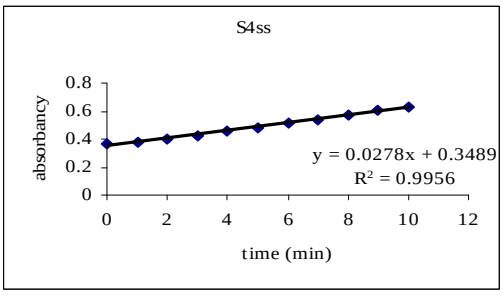






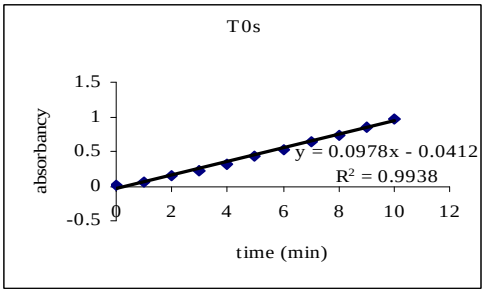


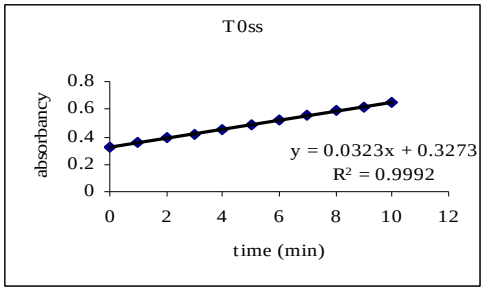


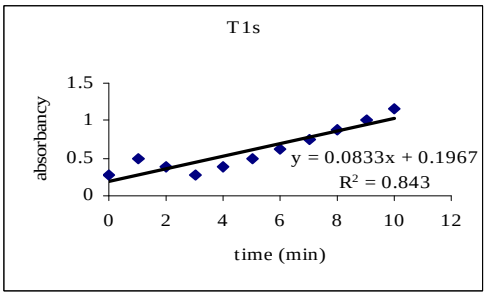


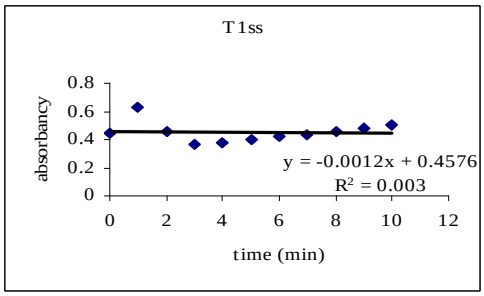
s. Glutathione absorbencies for patient S for the five treatments

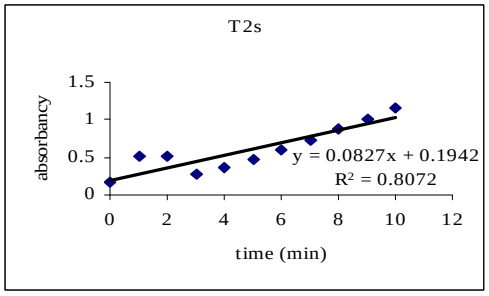
Appendix F (Continued)

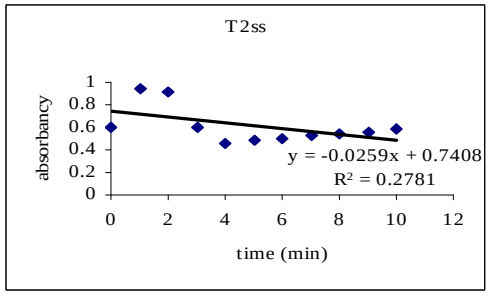


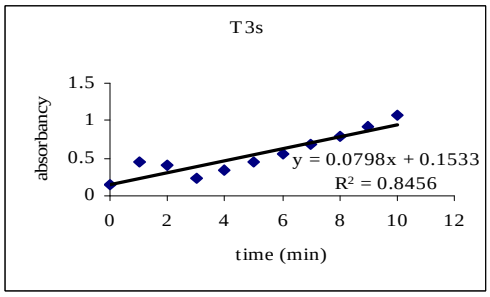


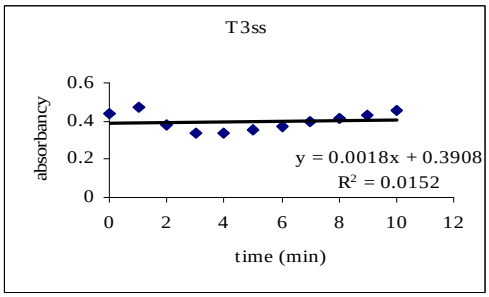


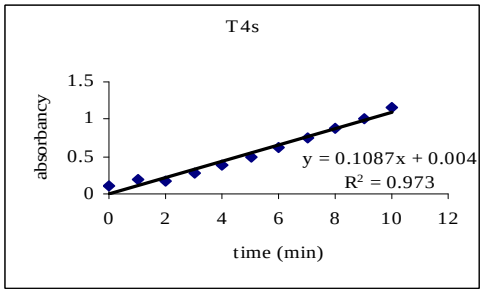


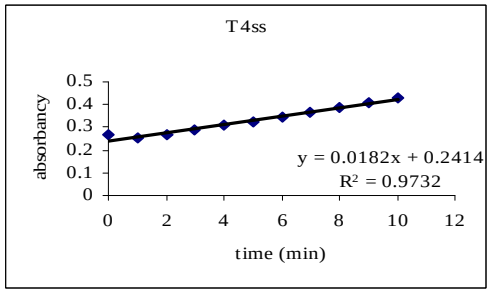












t. Glutathione absorbencies for patient T for the five treatments

About the Author

Daniela Sloan received a Bachelor's Degree in Ecology in 1996 followed by a Master's degree in Environmental Protection in 1997 from University of Bucharest. She received a second Master's degree in Marine Biology in 2003 from Georgia Southern University. She is first author on three publications on Echinoderms.