Novel Pretreatments of Whole Blood Using Fenton-like Processes for Trace Metal Analysis

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Abstract

Whole blood is a complex mixture of biological and chemical species. Its pretreatment, which is often conducted by dry ashing, is needed before the analyses of trace metals in whole blood. Recently photo-Fenton Advanced Oxidation Process (AOP) process has been used in the pretreatment of whole blood. Two new AOP processes using simple heating and microwave irradiation have been developed in the current work to pretreat blood samples. The treatments are based on a Fenton-like AOP with acid deactivation of the enzyme catalase. The first treatment is performed with a lab oven over 5 h, while the second uses microwave irradiation for 6 min. These methods allow for either cost-effective pretreatment through the use of the lab oven, or time savings through the use of the microwave oven. The degradations of blood and pure hemoglobin samples are compared through UV/visible spectroscopy, and the copper concentration in the treated samples were analyzed via anodic stripping voltammetry as a demonstration of analyzing trace metals in the pretreated whole blood.
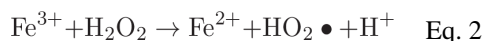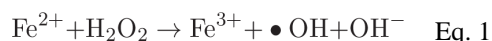
Keywords

Fenton-like Advanced Oxidation Process; pretreatment of blood samples; lab oven; microwave oven; electroanalysis of trace copper in blood; Biomedical Applications; Swine Blood

Introduction

Metal analysis in biological samples is an important and active area of study. Being useful within many fields, such as biomedical, wastewater treatment, environmental, and even veterinary studies, there will always be a call for improving the processes involved (Miller et al. 1993; Knahr, Karamat and Pinggera 2005; Lewis, O'Haver and Harnly 1984; Wang 1982). One of the most important steps is the pretreatment method, especially when analyzing metals such as copper or chromium that form complexes or are bound in macromolecules (Rodman, Carrington and Xue 2006; Yong et al. 2006). Currently, the most common pretreatment methods include dry ashing, chemical oxidation and advanced oxidation. In dry ashing, the sample is placed in an open vessel and the organics are destroyed through thermal decomposition under very high temperatures. This method can process large volumes of samples, but is time consuming and has increased error due to the required amount of samples (Mester and Sturgeon 2003; Bragg and Xue 2011). Chemical
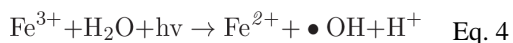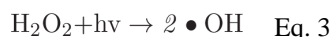
oxidation methods are often used in processes like wastewater treatment, but can require large quantities of oxidizing agents such as hypochlorite and potassium permanganate, which can be costly and environmentally damaging (Szpyrkowicz, Juzzolino and Santosh 2001).

Advanced oxidation processes (AOPs) are a sample treatment method to destroy organics through the formation of highly reactive hydroxyl radicals (•OH) (Parsons 2004; EPA 1998; Legrini, Oliveros and Braun 1993; Symons and Worley 1995). The Fenton Process is a common AOP that uses a ferrous ion (Fe^{2+}) alongside H_2O_2 to form hydroxyl and hydroperoxyl radicals, which become the oxidants in the decomposition of organic compounds. The radical forming reactions involved in the Fenton Process are given below.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \quad \text{Eq. 1}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2 \bullet + H^+ \quad \text{Eq. 2}$$

In Eqs. 1–2, Fe^{2+} acts as a catalyst, cycling between the 2+ and 3+ oxidation states (Yong et al. 2006; Bragg, Armstrong and Xue 2012; Blanco and Torrades 2012).

The Fenton process does not require irradiation. However, if irradiation is introduced, the formation of radicals is greatly increased. This is known as the photo-Fenton Process and the increase in radicals is due to the direct hemolytic bond cleavage of the oxygen atoms in H_2O_2 as well as the regeneration of Fe^{2+} ions through the reduction of Fe^{3+} ions by light-induced electron transfer (Eqs. 3–4) (Parsons 2004; EPA 1998; Legrini, Oliveros and Braun 1993; Schulte et al. 1995).

$$H_2O_2 + h\nu \rightarrow \mathit{2} \bullet OH \quad \text{Eq. 3}$$

$$Fe^{3+} + H_2O + h\nu \rightarrow Fe^{\mathit{2}+} + \bullet OH + H^+ \quad \text{Eq. 4}$$

Three different reactions can take place between the hydroxyl ions and the organic species in solution (Eqs. 5–7):

1. Hydrogen abstraction (Parsons 2004; Zepp, Faust and Hoigne 1992)

$$HO \bullet + RH \rightarrow R \bullet + H_2O \quad \text{Eq. 5}$$

2. Electrophilic addition (Parsons 2004)

$$HO \bullet + RX \rightarrow HORX \bullet \quad \text{Eq. 6}$$

3.	Electron transfer (Parsons 2004)

$$HO\bullet +RX \rightarrow RX\bullet^{+} +HO^{-} \quad Eq. 7$$

As the hydroxyl and hydroperoxyl radicals attack any organic compounds in solution, more radicals are generated that continue to further decompose the organics. For this reason, photo-Fenton processes require small quantities of reagents, making them environmentally friendly alternatives to other methods (Parsons 2004; EPA 1998).

Past work in our group has successfully employed the photo-Fenton AOP method using UV irradiation as the photon source to mineralize swine blood samples (Bragg, Armstrong and Xue 2012). The study utilized a four-cell reactor that was designed and built in-house, and was coupled with the electrochemical detection of Cr(III) (trivalent chromium), an essential trace element in the body.

The work herein reports two separate Fenton-based methods using different irradiation methods. The first method employs a common laboratory convection oven: a novel, cost-effective form of blood pretreatment. The second reported method relies on irradiation from a synthesis microwave, which requires only six minutes to perform. The pretreatment methods have been further validated via UV-Vis spectroscopy, comparing pre- and post-treated sample spectra with those of pure hemoglobin. Furthermore, the copper concentration in the treated samples were analyzed via anodic stripping voltammetry (ASV), and compared with results using inductively coupled plasma-optical emission spectroscopy (ICP-OES).

Materials and methods

Chemicals and instruments

Sodium hydroxide (NaOH, Certified ACS, Thermo Fisher Scientific, Waltham, MA), potassium oxalate ($K_2C_2O_4$, Certified ACS, Thermo Fisher Scientific), ammonium iron(II) sulfate [$(NH_4)_2Fe(SO_4)_2\bullet 6H_2O$), Acros/Thermo Fisher Scientific], ethanol (95%, Decon Laborataries Inc., King of Prussia, PA), porcine hemoglobin (Sigma Aldrich, St. Louis, MO), whole swine blood (Wampler's Farm, Lenoir City, TN), nitric acid (HNO_3, 70%, Trace Metal Grade, Thermo Fisher Scientific) were used as received. Cu(II) ICP standard solutions (1000 mg L^{-1}, Sigma Aldrich) were diluted prior to use.

Prior to use, GCEs were polished to a mirror-like surface on a standard electrode polishing kit (CH Instruments, Inc., Austin, TX) including a 1200 grit CarbiMet™ disk, 1.0 and 0.3 μm alumina slurry on a nylon cloth, and 0.05 μm alumina slurry on a microcloth polishing pad. After polishing, GCEs were successively sonicated with deionized (DI) water, ethanol, and DI water again for 5 min each. Electrochemical measurements were carried out on a CHI 440a Electrochemical Workstation (CH Instruments). A three-electrode configuration consisted of a bare, unmodified GCE (3 mm in diameter, BAS Inc., West Lafayette, IN), Ag/AgCl (saturated KCl solution, CH Instruments) and a platinum wire (CH Instruments) as working, reference, and counter electrodes, respectively. Microwave irradiation was carried out using a Biotage 2.5 synthesis microwave in 20 mL microwave reaction vials. The oven

used was an Isotemp Standard Laboratory Oven (Thermo Fisher Scientific). UV-Visible spectra were collected using an Agilent 8453 photodiode array spectrophotometer and a 1.0 cm quartz cuvette. Blank spectra of deionized water were recorded and subtracted from those of the samples. pH measurements were carried out with a pH meter (Accumet Basic, Fisher Scientific).

Experimental procedures

Swine blood sampling

Whole swine blood was obtained from Wampler's Farm (Lenoir City, TN). The sample was taken from a single pig that was freshly slaughtered and placed in a 1-L Nalgene bottle. Prior to this, the interior of the bottle was coated with 2 g of $K_2C_2O_4$, an anticoagulant. Using ultrapure DI water and trace metal grade HNO_3, the blood was diluted to 5.00% and acidified to pH 3. This solution was used for following studies.

Catalase deactivation

Catalase is a natural enzyme that decomposes hydrogen peroxide and prevents the formation of hydroxyl radicals (Bragg, Armstrong and Xue 2012). In order for an AOP to reach its highest efficiency, the catalase must be inactive. Low pH (below 3.00) causes a temporary denaturation of catalase, allowing for the oxidation method to take place unimpeded (Yong et al. 2006; Bragg, Armstrong and Xue 2012). Once the catalase has been permanently destroyed, the pH is then raised without consequence if necessary. The pH must stay below 7 during the process. Otherwise the iron catalyst will precipitate as FeO(OH) (Shelor et al. 2011).

Laboratory-oven pretreatment

The optimized oven pretreatment method is carried out in a glass vial with a vented cap containing 15.0 mL of 5.00% whole blood (pH 3). Before placing it in the oven, 0.5 mg of $(NH_4)_2Fe(SO_4)_2\cdot6H_2O$ is added to the solution and the vial is warmed on a hotplate to ensure that it is fully dissolved. Prior to pretreatment, 1.00 mL of 30% H_2O_2 is added to the vial. The vial is then placed in the laboratory oven at 100 °C. After 1 h, 500 μL of 30% H_2O_2 is added, and the solution is left in the oven for another 4 h. The final pretreated sample which is a clear, yellow tinted solution is then removed from heat and allowed to cool to room temperature (Figure 1).

Microwave pretreatment

The optimized microwave pretreatment method is carried out in a 20 mL microwave reaction vial containing 15.0 mL of 5.00% whole blood (pH 3). The microwave vial can be sealed with an aluminum cap that can withstand pressures up to 30 bar. Before irradiation, 0.2 mg of $(NH_4)_2Fe(SO_4)_2\cdot6H_2O$ is added to the solution and the vial along with 1.00 mL of 30% H_2O_2. The vial is sealed and microwaved for 100 s at 150 W while being cooled with compressed N_2. The vial is then removed from the microwave and allowed to cool to room temperature before adding 500 μL of 30% H_2O_2. The vial is then re-sealed and microwaved again at 150 W for 160 s while cooling with compressed N_2. The final pretreated sample

which is a clear, yellow tinted solution is then removed from heat and allowed to cool to room temperature (Figure 2).

Results and discussion

Sample analysis

Using a laser and the Tyndall effect, the solutions were determined to be fully mineralized. This was further validated using a centrifuge and gravimetric analysis to ensure that no solids were left in the samples post treatment. Prior to voltammetric and ICP-OES analysis, the pH was increased to pH 7 by adding 1.0 M NaOH solution dropwise. At this pH, the majority of Fe ions crash out as insoluble FeO(OH) which were separated via centrifugation leaving a completely clear solution. The sample solutions were then diluted in a volumetric flask using ultrapure DI water.

ASV analysis of Cu in an oven-based AOP treated blood sample

Anodic stripping voltammetry (ASV) is a common analytical method for the determination of metals in solutions (Compton and Banks, 2011; Wang 1982 and 2003). By holding a working electrode at a very negative potential, metal ions in solution are reduced and electroplated onto the electrode surface. After a sufficient time of accumulating the metals onto the surface, the potential is swept from negative to positive, making the working electrode more anodic and stripping the metals off of the surface by oxidizing them back into an ionic state at their respective oxidation potentials. Through this oxidation, the stripping of a metal from the electrode surface produces a flow of electrons which can be analyzed in real time. This current is directly related the amount of analyte that was accumulated during the reduction step (Bard and Faulkner, 2001; Kemula and Strojek, 1963; Lin, Li and Mihailovi , 2015).

Copper analysis in the pretreated sample (pH 7) was conducted without the addition of a supporting electrolyte given that ions left in solution were sufficient. In this work, the ASV was paired with standard addition to determine the original concentration of copper in the original whole swine blood sample. The bare glassy carbon working electrode was held at −1.2 V for 300 s before sweeping the anodic sweep to 0.9 V using a frequency of 25 Hz, a step potential of 4 mV, and amplitude of 25 mV. The standard addition method was used to add Cu^{2+} standard to give concentrations in the range of 0.0–115.2 ppb (ppb = parts per billion) Cu^{2+}_{added} (Figure 3).

The voltammograms show oxidation peaks for both Cu^+ (−0.16 V) and Cu^{2+} (0.04 V). The calibration curves for both peaks are given in Figure 3. Using the linear equations for the Cu^+ and Cu^{2+} peaks (Figure 3), an original Cu concentration of 31.2 ppb and 30.8 ppb can be calculated, respectively, in the sample. The precision of the method was determined by calculating percent relative standard deviation (%RSD) for the highest concentration (the only concentration run as a multiplicate [n = 5]). The RSD is defined as the standard deviation divided by the mean for a set of replicate sample analyses; that is, the average deviation of values *relative* to the average of those values. The precisions for the Cu^+ and Cu^{2+} peak were determined to be 5.2% and 3.9% RSD, relatively. The average of these

concentrations translates to 1033 ± 9 ppb Cu in the original whole blood. This is calculated based on the known dilution factors: 15.0 mL of 5% whole blood was diluted to 25.0 mL and analyzed after mineralization.

ICP-OES analysis of Cu in an oven-based AOP treated blood sample

The calculated original concentration of Cu was further validated using ICP-OES. By creating a calibration curve using standards (Figure 4) followed by analysis of the sample solution, the Cu concentration was determined to be 32.9 ± 0.1 ppb, corresponding to 1097 ± 5 ppb Cu in the original whole blood. This concludes that the ASV analysis has a 5.8% error when compared to the ICP-OES data.

Analysis of samples via UV-Vis spectroscopy

UV-Vis spectra were taken of a diluted whole-blood solution acidified to pH 3, a solution made from porcine hemoglobin and acidified to pH 3, oven-based AOP treated blood at pH 3, and microwave-based AOP treated blood at pH 3 (Figure 5). The spectra illustrate the destruction of organics in the blood, as the hemoglobin peak at 380 nm no longer appears in the pretreated samples.

Discussion

Examination of the pretreated blood samples through naked-eye inspection and UV-Vis analysis indicates that the organics in solution have been destroyed. Both pretreatment methods produce solutions that are clear and pass the Tyndall-effect test. Although microwave-based AOP has been performed on blood samples, the samples are limited to 1 mL and require longer for complete pretreatment (Lee, Hur and Park 1998).

The direct analysis of the pretreated oven sample via ASV illustrates, in several ways, the success of this method. Since ASV is a sensitive method, organics in solution can easily interfere with the voltammetry, either by interacting with the electrode surface, or by inhibiting mass transfer of the analyte. Due to the clean, sharp peaks, these interferences are clearly absent. The fact that the voltammograms were smooth without the requirement of adding an electrolyte is another indication of complete, or near complete, sample mineralization.

Furthermore, the ability to detect the original Cu concentration by ASV with only 5.8% error, which was validated by ICP-OES, indicates that the majority, if not all, of the Cu was free in the solution. The Cu concentration in healthy pigs is ~1.1 ppm (parts per million) (Drouliscos, Bowland and Elliot 1970; Schultze, Elvehjem and Hart 1936), which agrees with the data we extracted through ASV and ICP-OES. More studies are required to validate these new methods, including reproducibility studies.

Conclusion

This work provides two novel methods for the pretreatment of whole blood. One utilizes a convection oven allowing for an inexpensive process, and the other is based on microwave irradiation for quick treatment. Analyses of the oven-treated sample using UV-Vis

spectroscopy, ASV, and ICP-OES all indicate that the method was successful. While the microwave-treated sample has not yet been analyzed by ASV, the UV-Vis spectra also indicate that the organics have been destroyed. Further studies will examine whether the 5.8% error between the ASV and ICP-OES data are caused by the AOP, sample preparation, or the analysis techniques themselves. It is the belief of the author that the two new mineralization procedures will open new avenues for research and medical laboratories.

Acknowledgments

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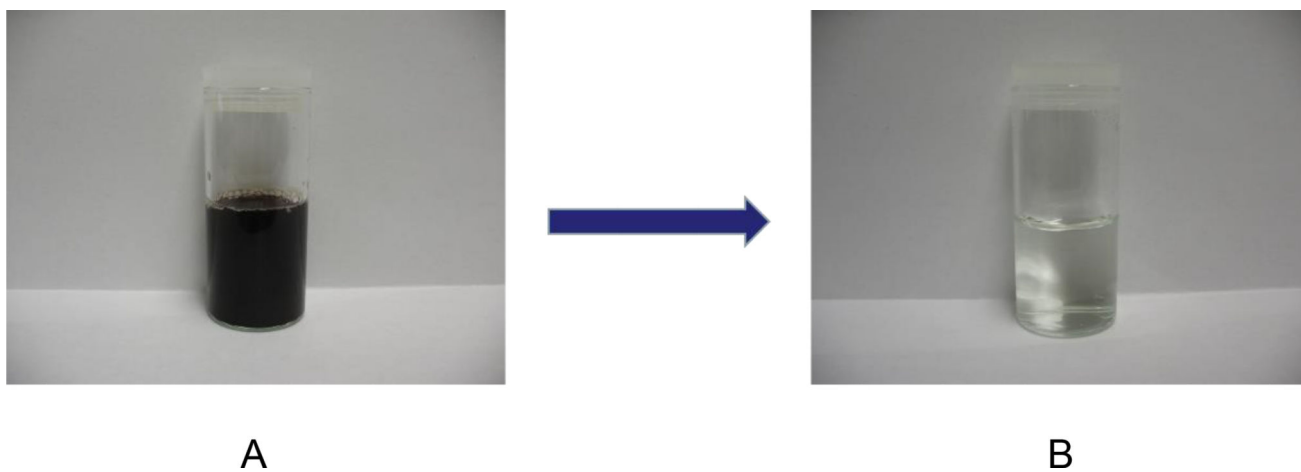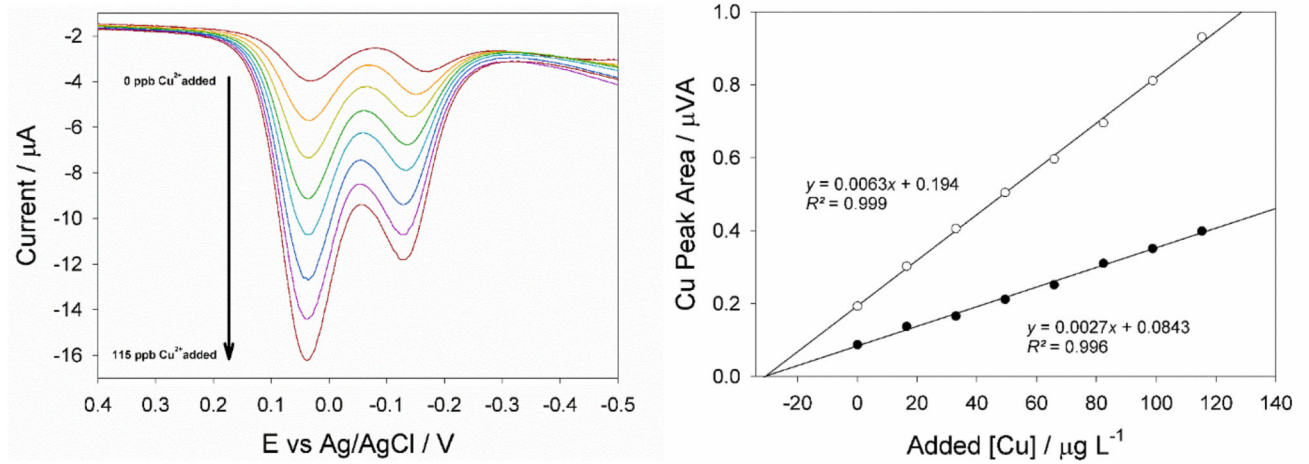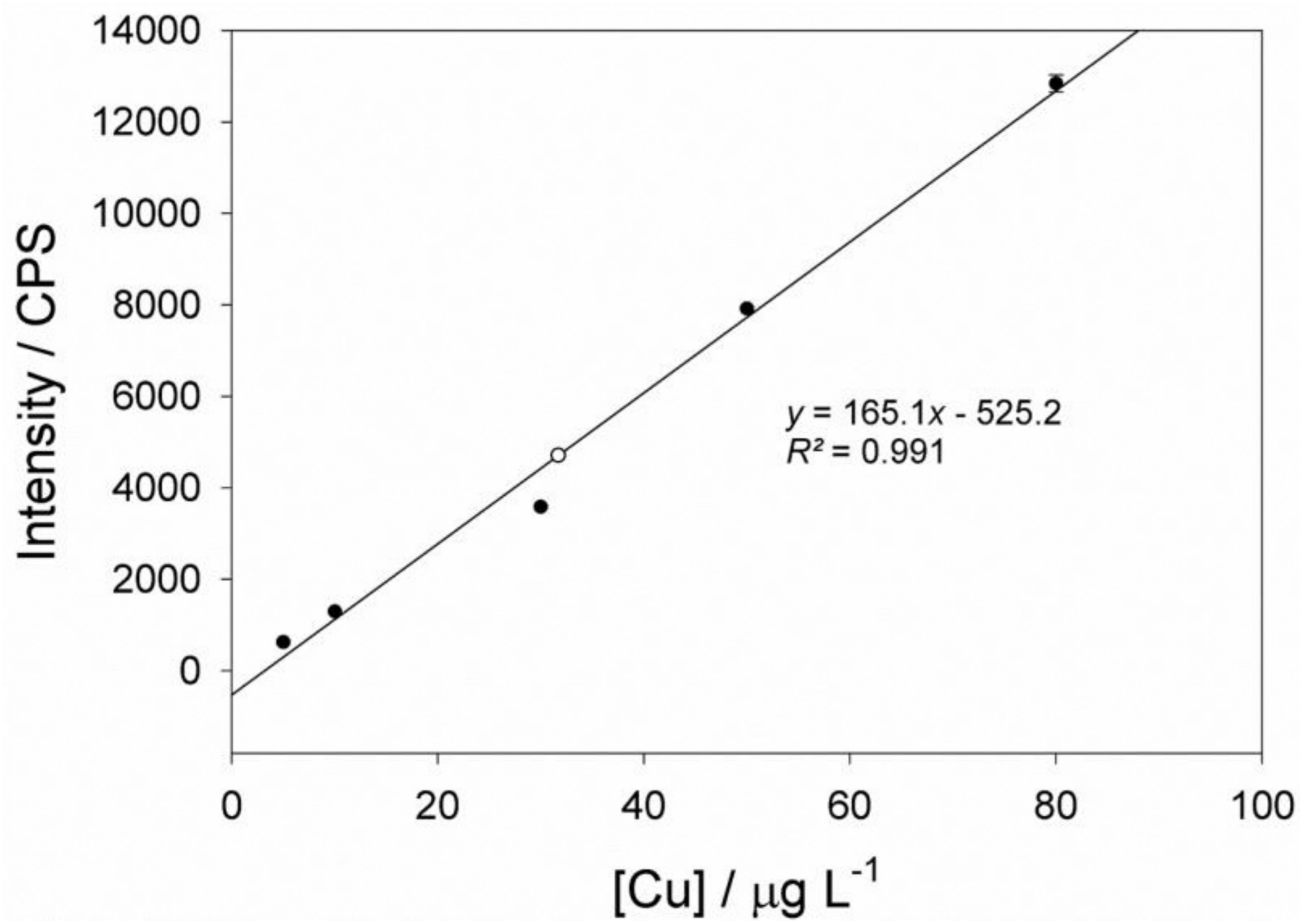
Figure 1.
Swine blood solution (A) before and (B) after oven-based AOP.

A   B
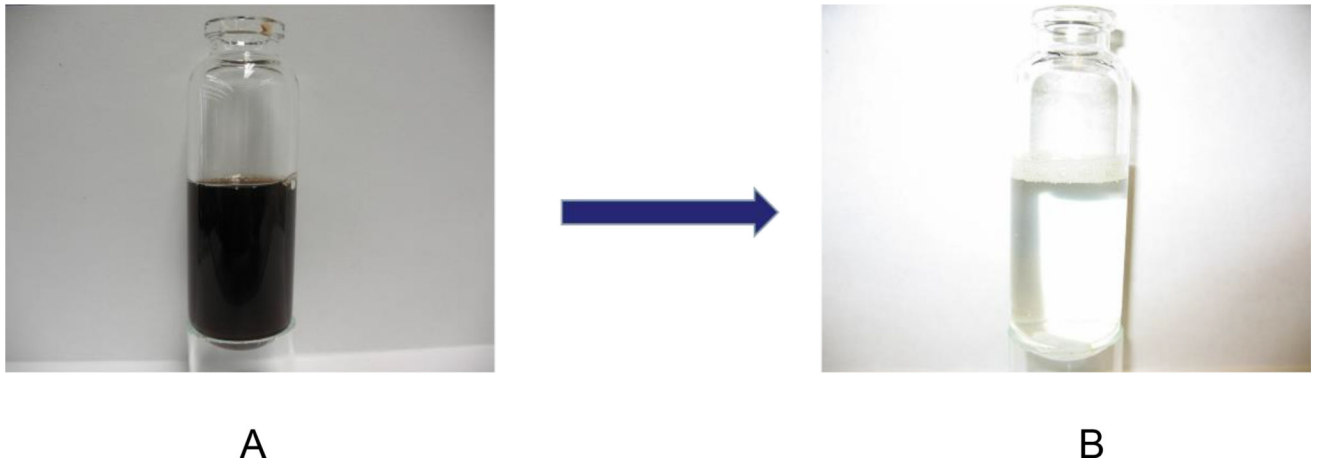
Figure 2.
Swine blood solution (A) before and (B) after microwave-based AOP.

Figure 3.
Voltammograms of Cu in an oven pretreated blood solution are shown on the left. Oxidation peaks for both Cu^+ and Cu^{2+} are observed. Calibration curves for the Cu^+ oxidation peak (black data points) and the Cu^{2+} oxidation peak (white data points) in pretreated blood are shown on the right.

Figure 4.
ICP-OES standard calibration curve of copper in pretreated blood ($\lambda = 327.393$ nm). The white data point was collected from the sample solution which was determined to be 32.9 $\pm$ 0.1 ppb.
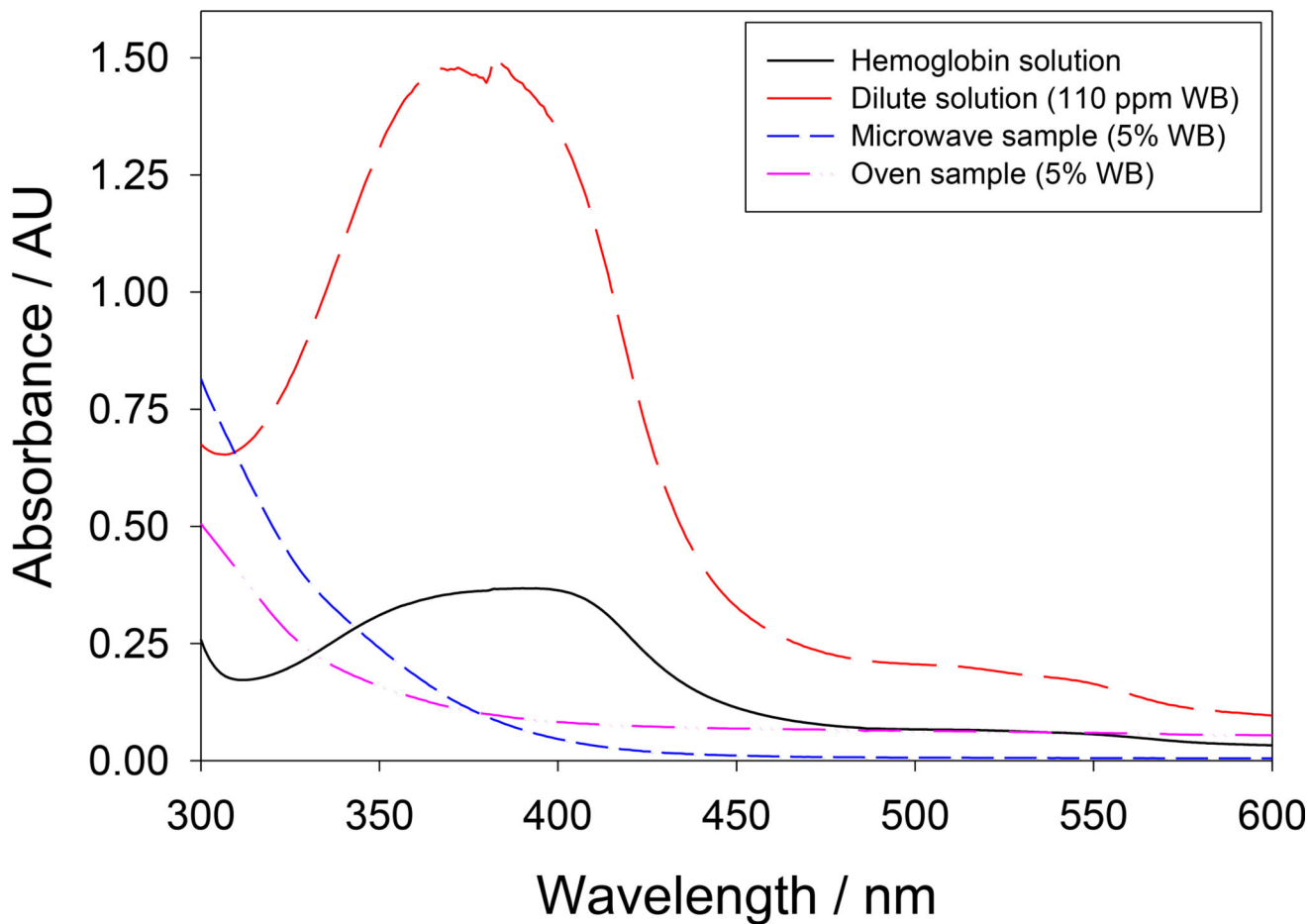
Figure 5.
UV-Vis spectra comparing oven- and microwave-based results with a dilute whole blood
(WB) solution, and porcine hemoglobin solution.