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# Disposable biosensor for detection of iron (III) in wines

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**Abstract:**

This paper reports the tuning of a fast, disposable, and label-free biosensor for quantification of iron (III) in food liquid samples such as wine. The biosensor is based on a field effect transistor(FET) where a net work of single –walled carbonnanotubes (SWCNTs) acts as the conductor channel, constituting carbonnanotubes field effect transistors (CNTFETs). An antibody such as transferrin with two specific high – affinity iron (III) binding sites, directly adsorbed to SWCNTs, was used as immunoreaction. Several individual CNTFETs were tested showing a linear range between 0.05 – 2 ng $mL^{-1}$ and a limit of quantification below 0.05 ng

$mL^{-1}$, much lower than previously reported analytical techniques. The mean coefficient of variation was 0.13% showing a low variability of the analytical response. On the other hand, it was not observed interference effect of zinc (II) ion at least until 1:4 iron - zinc ratio. Finally, recovery percentages of spiked wine samples were around 100%, showing the high accuracy of method. The main advantages of the devices developed are their speed, convenience (it is an economical method), and the avoidance excessive handling samples since they do not require further pre – treatment of samples.

## Introduction

Iron is a trace element that is present in food and human body with an average content around 3.8 g in men and 2.3 g in women. This element can be grouped into two categories in body compartments: heme and non heme iron. Heme iron is associated to hemoglobin and myoglobin consisting in an iron atom bounded by a porphyrin ring. On the other hand, non heme iron or inorganic iron could be present in the form of ferrous [iron (II)] and ferric [iron (III)]. This type of iron is less bioavailable than heme iron [1]. From a food technology view point, metals such as iron increase the fat oxidation rate promoting oxidative rancidity.

Currently, trace elements analyses in food are focused in determining the chemical form in which they are present (speciation analysis) rather than total quantification. However, iron is one of the most difficult elements in speciation analysis because iron species are presented in food in low concentrations, requiring the use of very sensitive methods [2]. In this sense, several methods have been proposed such as visible – spectrophotometric methods based on complex forming with 2-(5-bromo-2-pyridylazo)-5-(diethylamino)-phenol or bathophenantroline. They have been used for iron speciation in wines [3] and in soluble

fraction obtained from the in vitro digestion of food dishes [4]. To improve limits of detection and quantification in iron speciation, Inductively Coupled Plasma-Optical Emission Spectrometry (ICP – OES) and Mass Spectrometry (MS) methods coupled with liquid chromatography could also be used. Indeed, this methodology has been developed for the identification on non heme iron, heme iron and iron – containing myoglobin in meat and seafood [5, 6]. However, all above techniques do not avoid an excessive handling of samples. In addition, most of the methods used for iron speciation analysis in foods may modify the oxidation state of the element [7].

The use of disposable chemical sensors could avert all these drawbacks. They are economical in nature and designed to be one – shot, so they do not experience so – called memory effects, they do not require further pre – treatment prior to use or cleaning between measurements and they are versatile in different applications [8, 9]. Many of these sensors incorporate a substance having a high affinity for the element or molecule to be analyzed, thus allowing its quantification. These substances could be enzymes, antibodies, nucleic acids or whole cells among others [10]. The use of these devices in the determination of heavy metals has been summarized in a previous study [11].

Alongside this, incorporation of nanomaterials may enhance selectivity, sensibility and reproducibility of these devices thus allowing adequate miniaturization [12]. For example, single-walled carbon nanotubes (SWCNTs) are one-dimensional nanostructures which show high electron transfer reactions due to the presence of all carbon atoms in their surface [13]. Thus, their large surface area together with the possibility of functionalization with different molecules (such as antibodies of enzyme – linked immunosorbent assays, ELISA) allows them to be adequate sensors for the specific detection of various analytes [13, 14]. Field effect transistors with carbon nanotubes (CNTFETs) constitute a type of detection systems which use SWCNT as active elements.

Due to the affinity of the SWCNT with amino groups of antibodies [15, 16], the immobilization of an antibody specific on the SWCNT surface can be directly processed through a non-covalent adsorption, since this method preserves the structure of the SWCNT and consequently their mechanical and electronic properties [17]. Considering that the concentration of the antibody on the SWCNT surface, will be associated with the number of antibodies available for interaction with the target analyte, this latter concentration could be determined by the change of drain current ($I_d$) values before and after to analyte addition.

This type of devices has already been used, for example, in environmental applications, for the detection of atrazine and *Escherichia coli* [18, 19], in clinical analysis for the detection of human immunoglobulin E, human immunoglobulin G, C-reactive protein [20 – 22], as well as for food safety to the detection of *Aspergillus flavus* virus [23]. To carry on with this research line, the objective of the present study was to apply a CNTFET sensor to quantify iron (III) as iron species in red wine.



## Material and methods

### *2.1 Reagents*

SWCNTs synthesized through cobalt – molybdenum catalysis (CoMoCAT, Southwest Nanotechnologies), sodium cholate, Dulbecco´s saline phosphate buffer (PBS, pH 7.4), acetone, 1 – propanol and transferrin (2 mg $mL^{-1}$) were purchased from Sigma – Aldrich. All chemicals and solvents were of commercially available analytical reagent grade. Transferrin was diluted with 0.01 M phosphate buffer solution (PBS, pH 7.4) and stored at 4ºC.

### *2.2. Microfabrication of Field Effect Transistors (FETs)*

The microfabrication process was carried out in a clean room, a controlled and dust-free environment to avoid contamination of microfabricated devices (FETs), which were constructed in p-type silicon wafers (3 in. Ø, 0.5 mm, Sigma-Aldrich). Figure 1 displays the sequence of process steps and the final FET architecture. The silicon wafers were passivated with 400 nm of $SiO_2$ as an insulating layer by Plasma Enhanced Chemical Vapour Deposition. Then, thin layers of titanium (10 nm) and gold (100 nm) were deposited on $SiO_2$ through Physical Vapour Deposition by sputtering. The Ti/Au film was coated with a 1.5 µm layer of a photosensitive polymer called positive photoresist (PFR 7790G-27cP, JSR Corporation) by spin coating. The photoresist becomes soluble to ultraviolet light due to the breaking of polymer chemical bonds and the regions with the resist mask are thus defined, while the remaining surface is cleaned. The lithography process was used to define the source and drain metal contacts through the transfer of a geometric pattern (photomask) to the light-sensitive photoresist. As the whole substrate surface was exposed to the ultraviolet laser (inverted mask), the uppermost layer of the substrate that is not protected by photoresist was removed by ion beam etching using an automated ion milling system, and after removing the remaining photoresist on the surface of sensor structures, by immersing the wafer in a microstrip solution (Microstrip® 2001, Fujifilm) at 65ºC, the sensor structures were completely defined. The final step of FET microfabrication was the definition of the third contact (gate). For that, the wafers were coated with a photoresist film (1.5 µm) and the second mask (red layout with 150 x 150 $\mu m^2$) was defined by optical lithography. Then, 400 nm $SiO_2$ were removed by reactive ion etching in order to only an opening was defined in the resist, and 100 nm of Cr/Au were deposited by ion beam deposition. The lift-off was used to remove the remaining photoresist and the Cr/Au film on the surface of the wafer, only remaining the Cr/Au film of the opening, which functions as metallic layer of the third contact (gate). The final step to obtain individualized FET was the dicing of the wafers with an automatic dicing saw (DISCO DAD 321). Finally, FETs were washed with acetone and distilled water, and dried with

nitrogen, and they were mounted to a print circuit board (PCB) which was previously designed by AutoCAD software to allow the connection of the contacts (source, drain and gate) of each FET to the PCB strip. FETs were fixed on PCB with acrylate-based glue (super-glue) and they are bounded with aluminum wires (25µm Ø), which were protected by silicone (Elastosil E41, Wacker). For each FET, three chromium strips were defined and, at the end of them, a pin was soldered to make the connection in order to provide the electrical characterization of sensors in future experiments.

*2.3. Sensor surface functionalization*

Sensor surface functionalization was performed in two steps. Firstly, the FET surface was washed with acetone, 1 – propanol, desionized water and dried under $N_2$. Then, 2 µL of SWCNTs dispersion was deposited on the clean FET surface for 15 minutes at room temperature and also dried under $N_2$, in order to obtain the CNTFETs. The SWCNTs dispersion was prepared according to the optimized conditions reported in the work of Justino et al. [24], that is, through non-covalent functionalization of commercial SWCNT (~ 4 mg) with an anionic surfactant (14 mL of aqueous solution of 0.2 % w/v Sodium Cholate) after 60 minutes of sonication and 7 minutes of centrifugation at 2000 × g and 20 ºC.

A recognition molecule for iron (III) such as transferrin, was immobilized over the surface of the CNTFETs by dropping 2 µL of a transferrin solution to PBS at different concentrations (0.5, 1 and 2 µg $mL^{-1}$). The prepared devices were kept overnight under refrigeration (4°C).After adsorption of transferrin, the CNTFETs were dried with $N_2$ and electrically characterized the $I_D$ vs $V_D$ between 0 and +2 V.

*2.4. Iron (III) determination*

In order to detect iron (III), 2 µL de iron (III) solutions at different concentrations from 0.05 to 2.5 ng $mL^{-1}$were dropped into six individual CNTFETs per concentration with two replicates (total of 10 CNTFETs) and incubated 15 minutes at room temperature. CNTFETs were dried under $N_2$, and their electrical characterization was immediately processed. The electrical characterization of the disposable CNTFETs was based on the assessment of their output characteristics (drain current ($I_D$) as a function of the applied drain voltage ($V_D$) at several gate voltages ($V_G$, from -5 and +5 V), and the $I_D$ value at $V_D$=+1V and $V_G$=+1V was extracted for each measurement. The reproducibility was evaluated performing all measurements in six individual FET devices. The analytical response ($\Delta I_{Iron\ (III)}$) was calculated by the difference between the $I_D$ obtained after transferrin measurement ($I_{TF}$) and the $I_D$ obtained after the transferrin – iron (III) measurement ($I_{TF-Iron\ (III)}$) at $V_D$=+1V and $V_G$=+1V.

**Results and discussion**

Transferrin is a transport glycoprotein of iron in the plasma, with two specific high – affinity iron (III) binding sites and a molecular mass between 70 – 95 KDa. It could be used as a biomarker to assess iron status in humans [25] or as antibody combined with nanomaterials to quantify ferric ion, which was the case of the present study. Transferrin may be considered a siderophore similar to hemoglobin, lactoferrin and ferritin. These molecules are powerful complexants of iron (III) being secreted into the extracellular medium by a large number of unicellular organisms [26]. The binding of each ferric ion to transferrin is accompanied by the release of three protons and the binding of an anion derived from carbonic acid [27]. The ferric-siderophores (iron (III)-chelate complexes) are then assimilated by the cells via a specific receptor of the complex. Once inside the cell, the iron is released either by reduction to iron (II) (for which the siderophore has scarce affinity) or by hydrolysis of the ferric-siderophore accompanied by release of its iron [26].

To find the best option, several concentrations of transferrin solution in PBS (0.5, 1 and 2 μg $mL^{-1}$) were tested. The lowest concentrations (0.5 and 1 μg $mL^{-1}$) were inefficient for iron quantification because they did not show a straightforward relation between physical property measurement and element recognition. Probably because of these low concentrations (<1 mg $mL^{-1}$) all binding sites of transferrin are saturated by iron (III) and cannot be quantified the amount of analyte bound related with a signal transfer. Therefore, all the assays hereinafter were made with the highest transferring concentration in PBS (2 μg $mL^{-1}$).

The performance of the proposed biosensor was evaluated by determining ferric ion solutions in deionized water at different concentrations (0.05, 0.1, 0.5, 1.5, 2 and 2.5 ng $mL^{-1}$) at $V_G$ = +1 V and $V_D$ = +1 V for six individual CNTFETs for each concentration (and two replicates). For all samples, the $I_D$ obtained after transferrin – iron (III) measurement ($I_{TF-Fe}$) was lower that the $I_D$ obtained after transferrin measurement ($I_{TF}$), which means that the analytical signal ($\Delta I_{Iron\ (III)}$) is negative. As long as the iron (III) concentration added is higher, the decrease of $\Delta I_{Iron\ (III)}$ is larger. Thus, saturation of transferrin binding sites by iron (III) produces this drop in electric current. The variation of the analytical response ($\Delta I_{Iron\ (III)}$) obtained with CNTFETs for iron (III) concentrations studied is shown in Fig. 2. According with transferrin concentration used as antibody in the biosensor tested (2 μg $mL^{-1}$) and with the two specific binding sites for iron present in transferrin molecule, it could be stated that transferrin is saturated when the iron (III) concentration is around 2.5 ng $mL^{-1}$ as it can be seen in Figure 2.

The coefficients of variation (CV) calculated from the standard deviation (for the 6 CNTFET per concentration) were 0.26, 0.03, 0.12, 0.10, 0.12 and 0.13% between 0.05 and 2.5 ng $mL^{-1}$, with an average CV of 0.13%, which shows a low variability of the analytical response. There was no statistically significant difference ($p < 0.05$) between various CNTFETs for the same concentration of iron (III). In return, there were statistically significant differences ($p <$

0.01) between the different levels of iron concentrations, which means that CNFETs respond differently to each iron (III) concentration.

In Figure 3, the calibration curve was displayed, where a linear calibration equation within the concentration range of 0.05 – 2 ng $mL^{-1}$, correlating iron (III) concentration ($c_{Iron\ (III)}$ in ng $mL^{-1}$) to difference in electric current ($\Delta I_{Iron\ (III)}$), was $\Delta I_{Iron\ (III)} = 31.09\ c_{Iron\ (III)} - 0.172$, with a (squared) linear correlation coefficient of $R^2$ of 0.975.

The limits of detection and quantification found for the biosensor studied are below 0.05 ng $mL^{-1}$. Many of the analytical techniques used for iron (III) speciation are spectrophotometric methods based on absorbance measurements at a specific wavelength of a complex formed between a chelating agent and iron (III). These methods, besides implying a greater sample handling, achieve higher limits of detection and quantification (Table 1). Thus, Krzek et al. [28] suggest an UV spectrophotometric method for iron (III) determination in medicines with ammonium thiocyanate. The detection limit and quantification limit for this method was 0.09 μg $mL^{-1}$ and 0.26 μg $mL^{-1}$ respectively. Similarly a colored compound, formed in a specific reaction between squaric acid and iron (III), has been used to quantify this ion by UV – visible spectroscopy with a detection limit of 0.3 μg $mL^{-1}$ [29]. Utilization of fluorescence spectroscopy and ICP – MS techniques allow obtaining improved limits. Iron (III) has been determined in waters using flow injection fluorescence and salicylic acid with a limit of quantification of 1.12 ng $mL^{-1}$ [30]. In the same way, limit of detection of 0.60 ng $mL^{-1}$ has been obtained for iron (III) in a solid phase extraction procedure following ICP – MS determination [31]. However, these methods do not avoid possible excessive samples handling, being also expensive and not commonly present in all chemical labs.

To evaluate the potential interference of other metal ions, a foreign metal ion such as zinc (II) were added to a series of standard working solutions contanining 0.5 ng $mL^{-1}$ iron (III). Iron and zinc interactions in human diets have been widely described. Several hypotheses have

been postulated based on iron and zinc uptake studies in hepatocytes, oocytes and fibroblasts arguing that iron and zinc compete for the same influx transporter [32]. In our study, whatever of the iron (III) – zinc (II) ratios studied did not found interference effect on the ability of the CNTFETs to detect iron (III) concentrations in the presence of zinc (see Table 2). Thus, a signal-response corresponding to a iron (III) concentration of 0.5 ng $mL^{-1}$ was obtained even when zinc (II) concentration is four times higher, showing that zinc (II) ions are not chelated by transferrin instead of iron (III). The decrease in signal observed in 1:1 ratio case, must be attributed to a slight defect in the devices used in this assay (which was not observed in other experiments) and not to a potential interference between zinc (II) – iron (III).

Finally, in order to validate the accuracy and precision of the proposed method in the analysis of liquid foods, wine samples with iron (III) standards added were analyzed. Recovery percentages of spiked samples are shown in Table 3. It can be observed that recovery percentages were around 100%, showing the accuracy of method. Due to high concentrations of iron (III) in wine, samples were submitted to a dilution 1: 10000. The necessity of sample dilution to achieve the working range of 0.05 – 2 ng $mL^{-1}$ could be a drawback of the method when iron (III) concentration is high. However, even in this case, it is avoided treatment sample steps such as dry or wet mineralization, required in atomic absorption spectroscopy methods. Thus, CNTFETs fabricated in this work can be used to detect ferric ion in food samples liquids as a simple, label – free, and disposable methodology.



## Conclusions

The present work tunes up a disposable sensor to quantify ultratrace amounts of ferric ion in water and liquid food samples such as wine. This sensor is able to detect iron (III) concentrations in a linear range between 0.05 – 2 ng $mL^{-1}$ and a limit of detection and

quantification below 0.05 ng $mL^{-1}$, much lower than many analytical techniques. Furthermore, it presents a high response capacity to different iron (III) concentrations and simultaneously a low variability of the analytical response to same iron (III) concentration. In addition, wine analyses could be performed in a short time, no more than 15 min, avoiding possible excessive samples handling and pre - treatment steps which are required in other techniques. Finally, speciation analyses of iron (III) could be made in a less expensive way than other reported analytical methods. Thus, CNTFETs can be used to quantify iron (III) in wine samples as a simple, label – free and disposable methodology.



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Figure 1. Schematic 1) cross views and 2) top views of microfabrication steps of FET: a) SiO2 and Ti/Au depositions, b) Coating with photoresist, c) Lithography (inverted mask, resist mask defining the contacts), d) Ion milling Ti/Au films to remove undesired zones, e) Resist strip, f) Coating with photoresist, g) Lithography for opening contact via (noninverted mask, resist all over, except in 150x150 $\mu m^2$ holes), h) Reactive ion etching of $SiO_2$ , i) Metallic layer deposition, j) Lift-off, and k) final FET architecture.

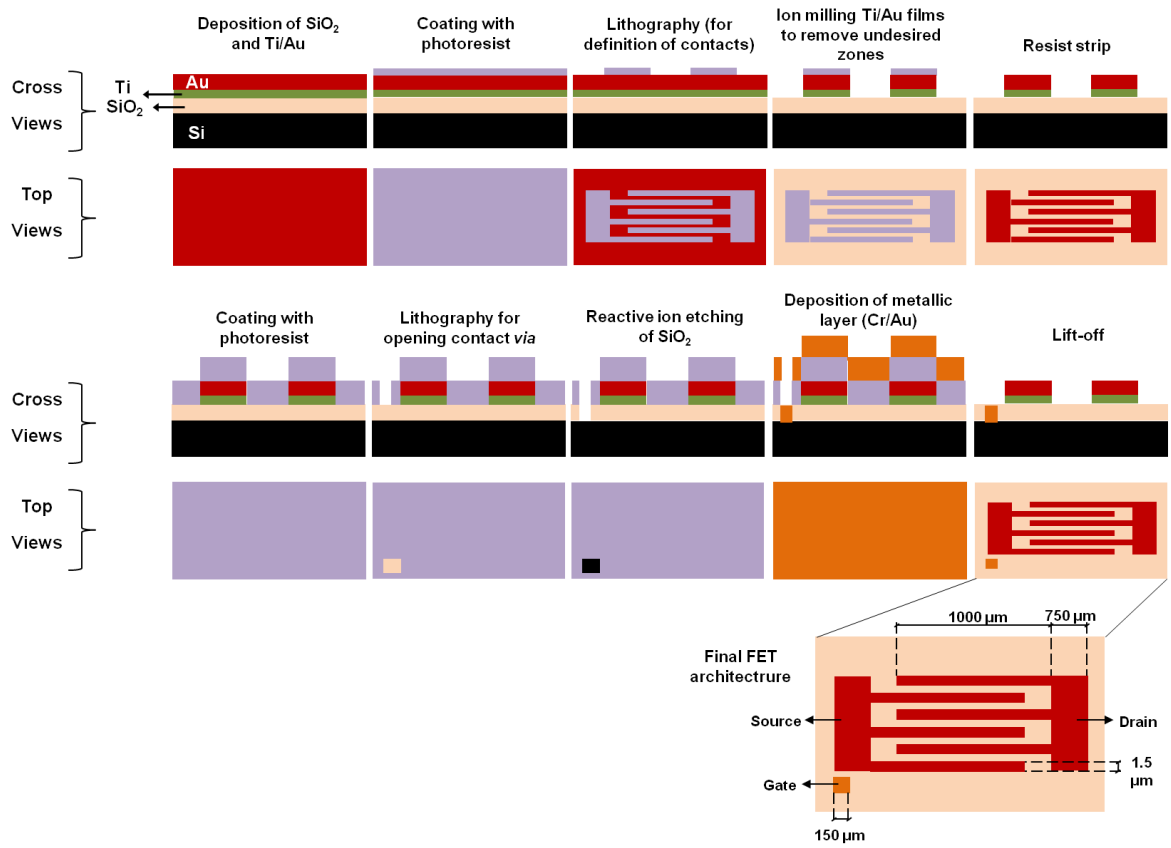



Figure 2. Variation of the analytical response (mean response of six individual CNTFETs) obtained at different concentrations of iron (III) from 0.05 to 2.5 ng $mL^{-1}$ at $V_G$ = +1 V and $V_D$ = + 1 V.

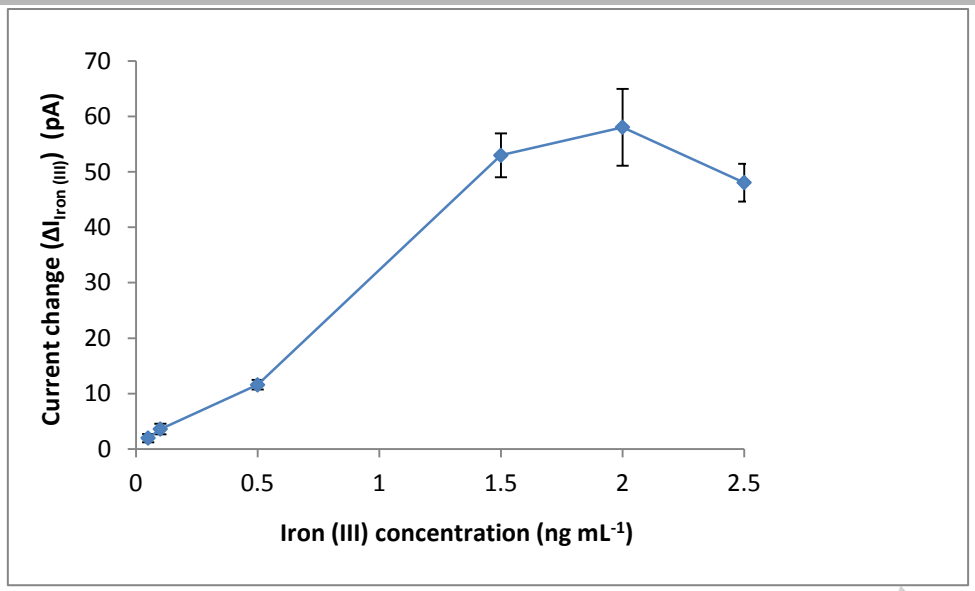
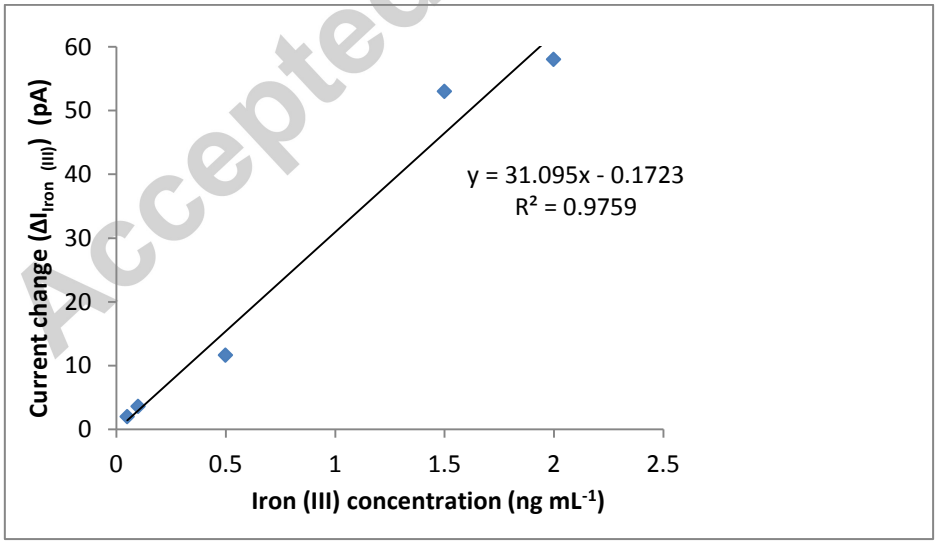



Figure 3. Calibration curve of CNTFETs to iron (III).



'Graphical Abstract (for review)

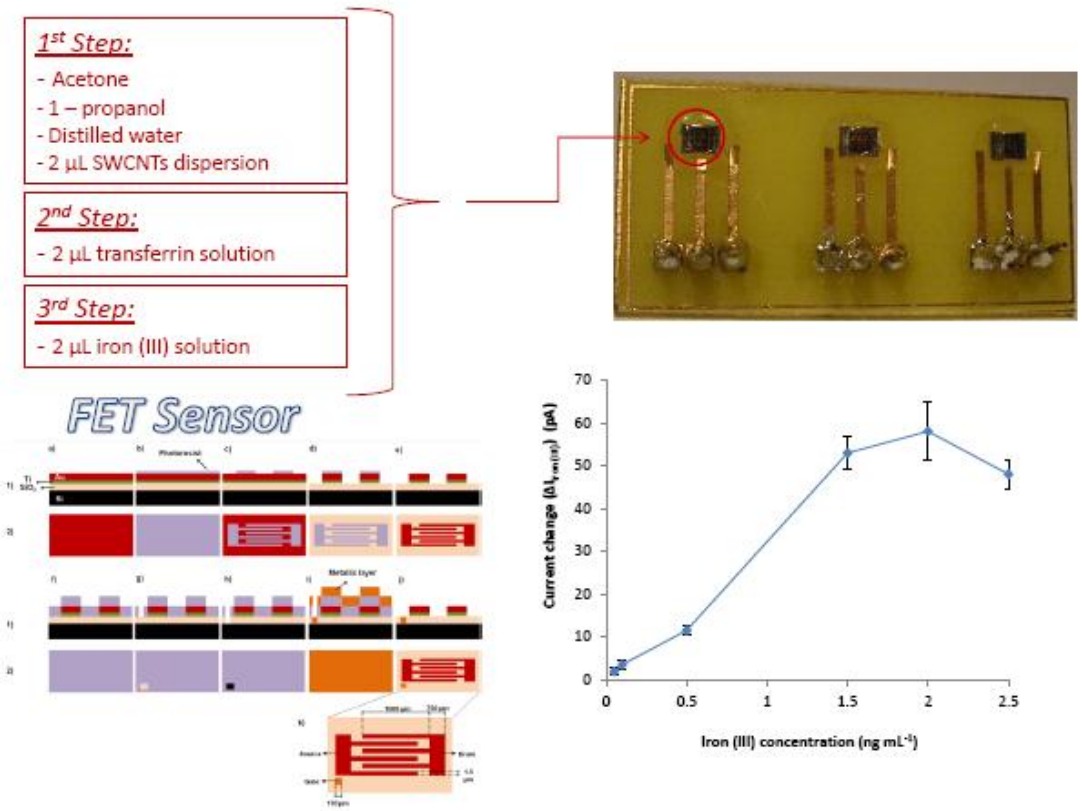



Table 1. Comparison with other reported methods for iron (III) analysis

| Method | Working range | Limit of detection | Reference |
|---|---|---|---|
| UV – visible spectroscopy | 0.87 – 2.31 µg $mL^{-1}$ | 0.09 µg $mL^{-1}$ | [28] |
| UV – visible spectroscopy | 0.5 – 20 µg $mL^{-1}$ | 0.3 µg $mL^{-1}$ | [29] |
| Flow injection fluorescence | 5 – 100 ng $mL^{-1}$ | 0.3 ng $mL^{-1}$ | [30] |
| Solid phase extraction + | 80 – 200 ng $mL^{-1}$ | 0.60 ng $mL^{-1}$ | [31] |

| | | | |
|---|---|---|---|
| ICP - MS | | | |
| Electrochemical immunsensor | 0.05 – 2 ng $mL^{-1}$ | < 0.05 ng $mL^{-1}$ | This work |

Table 2. Effect of potentially interfering ions in the analytical response of the CNTFETs

| Iron (III) standard concentration (ng $mL^{-1}$) | Zinc (II) standard concentration (ng $mL^{-1}$) | Zn (II) / Fe (III) ratio | $-\Delta I_{Iron\ (III)}$ (pA) | Found concentration (ng $mL^{-1}$) |
|---|---|---|---|---|
| 0.5 | 0 | - | 10.4 ± 5.6 | 0.3 ± 0.2 |
| 0.5 | 0.5 | 1 : 1 | 4.9 ± 0.5 | 0.16 ± 0.02 |
| 0.5 | 1 | 2 : 1 | 12.1 ± 3.4 | 0.4 ± 0.1 |
| 0.5 | 1.5 | 3 : 1 | 13.3 ± 6.4 | 0.4 ± 0.2 |
| 0.5 | 2 | 4 : 1 | 14.6 ± 1.7 | 0.48 ± 0.06 |

Table 3. Recovery of the CNTFETs in red wine samples.

| Spiked concentration of Iron (III) (ng $mL^{-1}$) | $-\Delta I_{Iron\ (III)}$ (pA) | Found concentration (ng $mL^{-1}$) | Mean recovery (%) |
|---|---|---|---|
| 0.0 | 1.8 ± 0.1 | 0.063 ± 0.009 | - |
| 0.5 | 18.6 ± 3.4 | 0.6 ± 0.1 | 108 |
| 1.5 | 51.1 ± 16.3 | 1.6 ± 0.5 | 106 |

## Highlights

Biosensor based on field effect transistor was developed for detection of iron (III)

Biosensor is able to detect iron(III) in wines at low concentrations

The avoidance excessive handling samples is one of the advantages of the biosensor