ABSTRACT:
The present invention discloses an electrochemical method to determine the presence of formaldehyde in food samples, for example fish and other seafood products by way of an enzymatic-based sensor. The method includes contacting the sample with an amperometric sensor, which comprises an electrode coated with an immobilized enzyme and measuring the current changes as the output signal at a constant voltage, which indicates the presence of formaldehyde in the sample.

Figure 1
Published:
— without international search report and to be republished
upon receipt of that report (Rule 48.2(g))
DETECTION OF FORMALDEHYDE

This present invention relates to a method for determination of formaldehyde in food samples. More particularly, the present invention relates a simple, rapid and sensitive electrochemical method for determining the presence of formaldehyde in fish and other seafood products by way of an enzymatic-based sensor.

BACKGROUND OF THE INVENTION

Formaldehyde (FA) is often used in fishery industry as preservative to maintain its freshness and prevent microbial spoilage. However, it is harmful for human consumption when used in excess as the residues retained in the fish muscles although it has been cooked, roasted or boiled (Kolodziejska et al., 1994; Sikorski et al., 1976). Besides, it can induce cancer and has been classified "as carcinogenic to humans" by International Agency for Research on Cancer (IARC) in the Group 1 (Bianchi et al., 2007). As established by Food Regulation 1985, formaldehyde content should not be more than 5 mg/kg and this level must be monitored strictly.

Several methods such as Nash method (Benchmann, 1996), HPLC (Bianchi et al., 2005), GC-MS (Bianchi et al., 2007), bio-sniffer (Mitsubayashi et al., 2008) and electronic nose (Zhang et al., 2008) have been used for determination of formaldehyde. Nevertheless, these methods involve long hours, toxic reagents and the determination of formaldehyde can be interrupted from a number of interferences and result in biases of analysis as well as not suitable for real-time measurements.

Currently, electrochemical analysis has attracted attention of many researchers to develop methods for analysis purpose as it offered the desired criteria. Biosensors such as potentiometric, conductometric, capacitance and amperometric based have been applied in various fields and also been commercialized.

The methods usually integrate with biorecognition element such as enzyme as the biocatalyst in the redox reaction. The properties of the enzyme are unique where the active site of the enzyme can distinguish the specific substrate to react with. However, enzyme is unstable in solution and required polymers to hold it from...
denaturation as well as extent its shelf life. Therefore, there is a need to immobilize the enzyme in a matrix to improve the enzyme properties and also the biosensor performances.

From the previous literatures, Korpan et al., 2000 and Hasbullah et al., 2003 described a method for determination of formaldehyde by using potentiometric incorporated with alcohol oxidase (AOX) which highly depends on pH, buffer capacity and ionic strength. Viaello et al., 1996 demonstrated the usage of NAD-dependent formaldehyde dehydrogenase (FDH) by using ion-sensitive-field-effect transistor but it required enzyme and cofactor to be added in bulk solution.

Dzyadevych et al., 2001 described the determination of formaldehyde by conductometric biosensor which utilized AOX which not selective to formaldehyde in the presence of methanol in the mixture. While Vianello et al., 2006 used FDH immobilized in Eupergit-C and ECH-Separose-4B which required a long procedure. Ben Ali et al., 2006; 2007 described capacitance concept to determine formaldehyde by using FDH (independent and dependent glutathione enzyme) immobilized in Nafion polymer and used gold electrodes$\text{SiO}_2$/Si/$\text{SiO}_2$/Ti/Au and electrolyte-insulator-semiconductor Si/$\text{SiO}_2$ (EIS) structures as the transducer. However, this method required substrate to be added in bulk volume.

Herschkovitz et al., 2000 demonstrated amperometric method integrated with FDH immobilized in Os(bpy)$_2$-poly(vinylpyridine) (POs-EA) which involved a few steps before it can be used. Nikitina et al., 2007 described bi-enzyme biosensor (NADD-glutathione dependent FDH and diaphorase) immobilized in osmium complex-modified redox polymer which definitely required highly cost as the enzyme is an expensive material. Shimomura et al., 2008 had also described immobilization of FDH in mesoporous silica materials which required a long time of preparation.

Usage of nanoparticles such as cadmium sulfide (Curri et al., 2002) and carbon nanotube (Selvaraj et al., 2009) in formaldehyde determination have been also described to improve the detection performance. However, no validation studies in real samples were performed.
Thus, there is a need to provide an electrochemical-based method that is simple, rapid and sensitive in determining the presence of formaldehyde in food samples.

SUMMARY OF THE INVENTION

In accordance with one embodiment of the present invention, a method for detecting formaldehyde in a food sample is provided. The method comprises contacting the sample with an amperometric sensor, the sensor comprising an electrode coated with an immobilized enzyme and measuring the current changes as the output signal at a constant voltage, which refers to the presence of formaldehyde in the sample.

The enzyme is preferably formaldehyde dehydrogenase immobilized in Nafion polymer through entrapment technique by drop coating method on the surface of the electrode. The NAD$^+$ dependent formaldehyde dehydrogenase acts as a receptor which is very selective to formaldehyde and retains its specificity to formaldehyde in the presence of interferences, such as methanol and ethanol.

The Nafion polymer is a synthetic sulfonated tetrafluoroethylene based fluoropolymer-copolymer. To solve the problem of enzyme stability that have been reported in the art, immobilization of the enzyme is performed in the ion-conducting Nafion polymer, which possess excellent chemical stability and high affinity toward large organic cations. In addition, the polymer also decreases the influence of buffer concentration and increases the response range of formaldehyde determination, which increases its sensitivity.

The sensor further comprises a counter electrode and a reference electrode. The counter electrode functions solely as the second half-cell and allows electrons to enter or leave the electrolyte as to complete the electrochemical cell circuit, while the reference electrode provides a stable electrochemical potential in the electrolyte.

In another embodiment of the present invention, a device is provided for detecting the presence of formaldehyde in a food sample. The device comprises a working electrode, a reference electrode and a counter electrode, the working electrode
coated with formaldehyde dehydrogenase enzyme immobilized in Nafion polymer to detect the presence of formaldehyde in the sample.

It is an advantage of the present invention to provide a simple, rapid and sensitive method and device for the determination of formaldehyde food samples such as fish and seafood products.

It is another advantage of the present invention to provide a method and device that does not require reagent materials, which may possess toxic effects.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a graph showing an example of differential pulse voltammetric output according to an embodiment of the present invention;

Figure 2 is a graph showing voltammogram of redox reaction of free and immobilized enzyme according to an embodiment of the present invention;

Figure 3 is a graph showing the effect of pH on the response of the sensor device according to an embodiment of the present invention;

Figure 4 is a graph showing the effect of enzyme loading on the response of the sensor device according to an embodiment of the present invention;

Figure 5 is a graph showing the linear response range of the formaldehyde sensor device according to an embodiment of the present invention;

Figure 6 is a graph showing the validation of formaldehyde results by using two different methods for ten days; and

Figure 7 is a graph showing the correlation of formaldehyde levels determined by the sensor device of the present invention with conventional Nash method.
DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method and device suitable for determination of formaldehyde in food samples, wherein formaldehyde dehydrogenase is used as the biorecognition elements. According to a preferred embodiment, the present invention comprises gold electrode as the working electrode, silver/silver chloride (Ag/AgCl) electrode as the reference electrode and platinum rod as the counter electrode.

Electrochemical study is carried out using Autolab III (Eco-chemie, Netherland) for differential pulse voltammetry (DPV) analysis. The potentiostat connected with DELL personal computer, which allowed the display of output of electrochemical system using General Purpose Electrochemical System (GPES) software.

Determination of formaldehyde is carried out by measuring the current changes generated from the redox reaction of the electroactive species as the product on the working electrode in the solution. The current response measurement is in proportional linear with the concentration of the analyte and produces whether normal dynamic range or error in the measurement. The reaction from the conducted study is then translated into current signal by the voltammetric analyzer and the data is transformed into a voltammogram for analysis.

The present invention is based on the reduction of NAD$^+$ to NADH as the key factor for determination of formaldehyde. The current changes of the reaction are obtained at constant voltage of -0.2 volt. The enzymatic reaction and its half-cell reaction equations were shown as below:

**Enzymatic reaction:**

\[
\text{FDH} \quad \text{HCHO} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{HCOOH} + \text{NADH} + \text{H}^+ 
\]

**Half-cell reaction:**

\[
\text{NAD}^+ + 2\text{H}^+ + 2\text{e} \rightarrow \text{NADH} + \text{H}^+ 
\]
In the reaction, formaldehyde dehydrogenase acts as electron transfer to facilitate the addition of one hydrogen atom to NAD\(^+\) and reduced it to NADH, whereas formaldehyde will be converted to formic acid.

The study of redox reaction is conducted in free and immobilized enzyme and it was found that the immobilized enzyme showed higher output signal than free enzyme indicating that immobilization of enzyme had advantage to support and enhance the catalytic activity of enzyme to occur maximally. By retaining its position with strong adherent, the recovery of the enzyme can occur from the substrate and product so that it can be used continuously. In addition, immobilization of enzyme minimizes denaturation of the enzyme during the operation and improves the stability of the enzyme and also gave effect to enzyme kinetic changes (Busto, 1998). Furthermore, Nafion polymer effectively acts as a protector to the coated electrode from fouling agent which existed in biological and food samples, and also prohibits anionic interferences.

In a preferred embodiment of the present invention, the enzyme loading ranges from 10 to 50 mg/mL of enzyme solution and NAD\(^+\) concentration from 0.01 to 2 mM. From the results, the optimum enzyme loading is 30 mg/mL and NAD\(^+\) concentration is 0.5 mM. The reaction time of the redox activity for determination of the formaldehyde is 40 seconds.

In addition, a response range study is also conducted with optimized parameters being applied and it was found that the sensor device of the present invention can determine the concentration of formaldehyde until 20 ppm. Calibration curve could be obtained from the linear response of formaldehyde which range from 1 to 10 ppm. The sensitivity of the sensor is 7.0264 nA ppm\(^{-1}\), which was determined from the calibration curve. The limit of detection is 0.016 ppm, which is the lowest concentration of formaldehyde that could be detected.

Interference study was also carried out to investigate the selectivity of the sensor device of the present invention by monitoring the capability of the sensor to distinguish feasible interferences with the interest substrate. It was found that the sensor retained its specificity towards formaldehyde and the addition of methanol or ethanol in the electrochemical system did not contribute to the response.
The present invention will now be described in further detail by way of non-limiting example.

EXAMPLE

Sample Preparation

Fresh Indian mackerel (Rastrelliger kanagurta) with diameter 20 cm was purchased from Sri Serdang wet market for this study. The fish sample was stored at a temperature of 4°C ± 1 for ten days and at every interval of two (2) days analysis of formaldehyde determination was performed. To perform this study, the fish was thawed and only the flesh was taken and cut into small portion. Then, 15 g of fish muscle was homogenized together with 30 mL of phosphate buffer (pH 8, 0.1 M) for 5 minutes. The homogenates were stored and were filtered with Whatman 1 filter paper each time to perform the analysis. The sample solution was used directly without further extraction and pretreatments. The determination of formaldehyde was conducted as described in the above description.

Preparation of Immobilized Enzyme

The enzyme solution was prepared by dissolving an appropriate amount of formaldehyde dehydrogenase in 0.1 M of phosphate buffer at different pH ranging from 6.5 to 8.5, with a preferred optimum pH 8. Immobilization of the enzyme was done by mixing the solution with Nafion polymer at different ratios (1: 10, 1:20, 1:30, 1:40 and 1:50) by retaining the amount of the enzyme volume as the enzyme is expensive to be used in vast quantity. The optimum ratio was found to be at 1:20. The mixture was then sonicated for 15 minutes to homogenize it and to make sure that the immobilization of enzyme in the polymer complete. After that, the mixture was stored at 4°C after or before being used to avoid the denaturation of the enzyme. The mixture was stable for more than six month and retains 90% of response.
Formaldehyde Determination

Before the electrode was used, the surface of the electrode was polished using alumina powder on a smooth cloth to ensure that the surface was cleaned from any contaminations. Then, the electrode was ultrasonicated in an ultrasonic bath for about 15 minutes. The immobilized enzyme slurry was then drop-coated on the electrode and dried.

After that, the electrochemical cell cap was fitted with three electrodes, namely the working electrode, the counter electrode and the reference electrode through the holes dipping in the electrolyte. The electrochemical cell was then connected to the voltammetric analyzer, potentiostat.

For the preparation of electrochemical electrolyte, 5 mL of phosphate buffer solution (25 mL deionized water was added to 0.68 g of potassium phosphate orthophosphate, pH adjusted by 0.1 M potassium hydroxide) was pipetted into the electrochemical cell. Then, 1 mL of standard formaldehyde (prepared from 100 ppm of formaldehyde stock solution) or sample solution was added and this mixture was purged with high-purity nitrogen gas for less than 1 minute to eliminate dissolved oxygen and to maintain a nitrogen atmosphere. This procedure was followed by addition of 0.5 mM NAD⁺ solution (prepared freshly) into the electrolyte system. Finally, the current measurement was recorded for formaldehyde content analysis.

In order to measure the current changes, "General Purpose Electrochemical System (GPES)" software was programmed in the computer. Mode of measurement which is differential pulse voltammetry was applied as the current changes measurement was done at small range of voltage, ranging from -0.4 to 0 volt.

The enzymatic reaction from conducted experiment was then translated into current signal by the voltammetric analyzer. Then, the data was transformed into a voltammogram for analysis which appeared either as cathodic (reduction) or anodic (oxidation) peaks. The determination of formaldehyde current changes was measured at -0.2 volt. An example of differential pulse voltammetric output is shown in Figure 1.
The study of different pH of supporting electrolyte involved immobilized enzyme which was coated on the gold electrode at three different concentrations of standard formaldehyde which were 1, 5 and 10 ppm.

While the study of enzyme loading was conducted at different concentrations of enzyme solution (10, 20, 30, 40 and 50 mg/mL) which then immobilized in Nafion polymer in a ratio of Nafion: formaldehyde dehydrogenase equals to 20:1. The current changes of the enzyme loading were obtained from DPV measurement.

Response range of formaldehyde biosensor was carried at different concentrations of standard formaldehyde solution (0.1 to 20 ppm). The calibration curve of formaldehyde biosensor was obtained from the linear range of the graph.

Validation of formaldehyde was carried out using two different methods which were formaldehyde biosensor and Nash method. Indian Mackerel fish was used as the sample and prepared as described on above. Determination of formaldehyde was measured at every interval of two days for ten days of storage at 4°C.

Results and Discussion

Based on Figure 2, the intensity of cathodic peaks for immobilized enzyme was higher than free enzyme for both methods. This demonstrated that, immobilization of enzyme had advantage to support and enhanced the catalytic activity of enzyme to occur maximally. By retaining its position with strong adherent, the recoveries of the enzyme can occur easily from the substrate and product so that it can be used repetitively.

PH played an important role in providing the appropriate environment for the enzyme to operate efficiently. However, different pH may cause the activation or denaturation of the enzyme as well as the enzyme activity significantly. As featured in Figure 3, it was found that at three different concentration of formaldehyde (1, 5 and 10 ppm), the optimum pH was at 8.0 and the usage of Nafion polymer as the membrane did not give significant effect to the alteration of pH.
For enzyme loading study (see Figure 4), it was found that 30 mg/mL concentration of enzyme exhibited maximal activity of enzyme by showing high current changes measurement. But it was gradually decreased at higher concentration of enzyme, as the aggregation and autodegraded of the enzyme itself was occurred due to the protein-protein interaction.

As presented in Figure 5, at the beginning of the response range was increased gradually until 10 ppm of formaldehyde and then became nearly constant at higher concentration. This phenomenon illustrated that the sensor has achieved its substrate diffusion capacity (coating) and saturation limit resulted from the biochemical constraint of the system. The enzymatic reaction was no further occurred efficiently as the kinetic rates became slower and produced similar response.

At initial day (see Figure 6), the amount of formaldehyde detected was low and then increased rapidly at 2 days storage. This phenomenon can be explained by the accumulation of formaldehyde resulted from the degradation of TMAO in the fish muscles catalyzed by TMAOase which contributed the denaturation of fish muscles from its native state. However, as the time storage increase after day two, the formaldehyde content was decreased gradually due to inhibition of the TMAOase by formaldehyde as it product achieved at its saturation level. The results determined from formaldehyde biosensor was comparable with Nash method.

**COMPARATIVE EXAMPLE**

For Nash method, the samples were extracted as previously reported by (Benchmann, 1996). Using Indian mackerel as the sample, the fish was stored as mentioned in above. The samples were cut into small piece and 15 g fish was weighed and homogenized with 30 mL of trichloroacetic acid (6%). Then, the homogenate was filtered using Whatman 1 filter paper and adjusted to pH 6-7 with KOH (15 % w/w) solution. The extracted aliquots were incubated in ice for about 30 minutes and used for formaldehyde determination using Nash method.

Nash reagent was prepared by dissolving 7.5 g of ammonium acetate, 0.15 mL of acetic acid, 0.1 mL of acetylacetone and adjusting the volume to 50 mL with
deionized water. The reagent was prepared fresh and used immediately. For determination of formaldehyde level, 2 mL of Nash reagent was added with 1 mL of sample solution prepared previously and was put in the test tubes and then incubated for 30 minutes at 37°C. Finally, the absorbance reading was measured at wavelength of 415 nm using spectrophotometer (Genesys 20).

Results

Referring to Figure 7, the results from both methods were found to be comparable with each other and yielded high correlation coefficient of 0.9982. From the statistical analysis, there were no significance differences ($p \leq 0.05$) between the two methods, which was calculated using student T -test.
CLAIMS

1. A method for detecting formaldehyde in a food sample comprising the steps of:
   (i) contacting the sample with an amperometric sensor, wherein the sensor comprising an electrode coated with an immobilized enzyme; and
   (ii) measuring the current changes as the output signal at a constant voltage, which indicates the presence of formaldehyde in the sample.

2. A method according to Claim 1, wherein the immobilized enzyme is formaldehyde dehydrogenase immobilized in an ion conducting polymer.

3. A method according to Claim 2, wherein the ion conducting polymer is a Nafion polymer.

4. A method according to Claim 1, wherein the current changes is measured by way of differential pulse voltammetric.

5. A method according to Claim 1, wherein the voltage of step (ii) is between -0.4 and 0 volt.

6. A method according to Claim 1, which is carried out at pH 6.5 o 8.5.

7. A method according to Claim 1, wherein the sensor further includes a counter electrode and a reference electrode.

8. A sensor device for detecting the presence of formaldehyde in a food sample, comprising a working electrode, a reference electrode and a counter electrode, wherein the working electrode is coated with an immobilized enzyme to detect the presence of formaldehyde in the sample.

9. A sensor device according to Claim 8, wherein the immobilized enzyme is formaldehyde dehydrogenase enzyme immobilized in an ion conducting polymer.
10. A sensor device according to Claim 9, wherein the ion conducting polymer is a Nation polymer.

11. A sensor device according to Claim 8, wherein the working electrode is a gold electrode.

12. A sensor device according to Claim 8, wherein the reference electrode is a silver/silver chloride (Ag/AgCl) electrode.

13. A sensor device according to Claim 8, wherein the counter electrode is a platinum electrode.
Figure 1

Figure 2
Figure 3

Figure 4
Figure 5

![Graph showing the relationship between concentration of formaldehyde (ppm) and current (nA).](image)

\[ y = 7.0264x - 401.2 \]
\[ R^2 = 0.9865 \]

Figure 6

![Bar chart showing concentration of formaldehyde over time (day).](image)

Legend:
- Nash method
- Formaldehyde biosensor
\[ y = 0.956x - 0.014 \]
\[ R^2 = 0.9982 \]

<table>
<thead>
<tr>
<th>Sample</th>
<th>Biosensor</th>
<th>Nash</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>3</td>
<td>0.28</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.58</td>
</tr>
<tr>
<td>5</td>
<td>0.91</td>
<td>0.88</td>
</tr>
<tr>
<td>6</td>
<td>1.42</td>
<td>1.32</td>
</tr>
</tbody>
</table>

**Figure 7**