Electrochemical Determination of Monocrotophos in Water and Fruits using acetylcholinesterase immobilized on CNTs modified Carbon paste electrode

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This study was conducted on the synthesis and electrochemical studies of immobilized acetylcholinesterase on chitosan and CNTs modified carbon paste electrode (AChE–CS/CNTs/CPE) for determination of monocrotophos (MCs) in water and fruits samples. For the preparation of the modified electrode, the functionalized CNTs were modified the CPE surface, and then AChE was immobilized through the mixture of AChE-CS on CNTs/CPE surface. Morphological analysis of modified electrodes using SEM showed that CNTs were homogeneously covered the CPE that forms a net-like film, resulting in the facilitation fixing of the AChE-CS onto the surface of GCE. The result of electrochemical studies using CV and DPV experiments indicated that the AChE-CS/CNTs/CPE can act as high stable, wide linear range and selective MCs sensor because of a combination of chitosan polymer and AChE, and high porous and more defect on functionalized CNTs as a nanostructured substrate which provides many electroactive sites on AChE-CS/CNTs/CPE. The sensitivity, linear range and limit of detection of the sensor were estimated 0.9038μA/μM, 1 to 70μM and 0.3nM, respectively. The practical application of AChE-CS/CNTs/CPE to the determination of MCs in prepared real samples of strawberry and agricultural wastewater was investigated and analytical results showed the acceptable values for recovery (98.70 to 99.77%) and RSD (1.77 to 4.08%). These results indicated a good accuracy of the proposed method for MCs detection in fruits and water samples.

Keywords: Acetylcholinesterase; Chitosan, Functionalized CNTs; Determination of monocrotophos; Differential pulse voltammetry

1. INTRODUCTION

Monocrotophos (MCs, dimethyl [(E)-4-(methylamino)-4-oxobut-2-en-2-yl] phosphate) as an alkenyl phosphate is a relatively cheap organophosphate insecticide [1, 2]. It is principally used as the fast-acting insecticide in agriculture to control weeds, insect infestation and diseases with both systemic and contact action against a wide range of pests on cotton, sugar cane, tobacco, potatoes,
peanuts, tomatoes, cucumber and ornamentals [3-5]. It is very toxic to animals and humans, as consequent it has been banned in the USA, EU and many countries.

It is reported that MCs can be absorbed into the animal and human body through inhalation, skin contact and ingestion and pesticide residues left behind on the food products [6, 7]. It is acutely toxic by all routes of exposure and put humans at an unusually high poisoning risk with headache, dizziness, blurred vision, tightness in the chest, sweating, nausea and vomiting, diarrhea, muscle twitching, convulsions, coma and death [8-10]. It is also used frequently as a common means of suicide.

Therefore, many studies have been performed for the detection and determination of MCs levels in agricultural products using high-performance liquid chromatography [11], gas chromatography [12], spectrophotometry [13], flow injection chemiluminescence method [14], chromogenic spray based method [15] and electrochemical technique [16]. Among these methods, electrochemical methods provide a useful platform for rapid, inexpensive and safer analysis of pesticides because of the capability of modification of electrode surface with a wide range of nanostructures and composites, thereby the sensing properties of electrochemical sensors can be promoted due to high surface-area-to-volume ratio, high chemical bioactivity and reactivity [17-19].

Therefore, this study was carried out for synthesis and electrochemical studies of AChE–CS/CNTs/CPE for determination MCs in water and fruits samples.

2. MATERIALS AND METHOD

2.1. Preparation the modified electrode

For the preparation of the carbon paste electrode [20], the homogeneous mixture of grinding paraffin oil (99.9%, Changshu Baihang Petrochemical Materials Co., Ltd., China) and graphite powders (90%-99.9%, Qingdao Furuite Graphite Co., Ltd., China) in a weight ratio of 3:7 was provided at 35°C. Then the obtained mixture was carefully pressed into a Teflon tube electrode (0.6 mm diameter), and then the surface of electrode was rubbed on the smooth weighing paper to get a smooth surface. Afterward, the prepared CPE was washed with deionized water.

For modification of the CPE with CNTs, the 1 g CNTs (99%, Guangzhou Hongwu Material Technology Co., Ltd., China) was functionalized in 20 ml mixture solution of 4.0 M HNO₃ (99.5%, Merck, Germany) and 10.0 M H₂SO₄ (>99%, Merck, Germany) in a volume ratio of 1:3. The CNTs were added to the acid solution and treated by magnetic stirring vigorously for 3 hours at room temperature [21]. Next, the functionalized CNTs were rinsed with deionized water several times. Subsequently, the functionalized CNTs were dispersed in a mixture of ethanol (99%, Shandong Aojin Chemical Technology Co., Ltd., China) and deionized water, and transferred into the oven at 70 for 5 hours to dry the functionalized CNTs. After cooling, a certain amount of functionalized CNTs were dispersed in N,N-dimethylformamide (DMF, ≥99%, Sigma-Aldrich, USA), and dropped on the surface of prepared CPE. After evaporating the DMF at room temperature, the modified CNTs/CPE was rinsed with deionized water.
For immobilization of AChE on CNTs/CPE [22, 23], the CNTs/CPE as the substrate was immersed in 5 μl of a mixture of AChE (≥99%, Sigma-Aldrich, USA) and chitosan (99%, Sigma-Aldrich, USA) in a volume ratio of 2:1 for 1 hour and dried overnight at room temperature. In order to remove any unbound AChE, AChE–CS/CNTs/CPE was carefully rinsed with 0.1 M phosphate buffer solution (PBS) pH 8.0. Next, the modified electrode was immersed in 5 μl of 0.5% Nafion (5%, Sigma-Aldrich, USA) as a protective layer for AChE–CS immobilized electrode and then stored in the fridge at 4 °C until use.

2.2. Real samples preparation

For the preparation of the real sample of fruits, strawberry samples were weighed and chopped, and then their juices were taken. The juice was transferred to centrifuge tubes at 1500rpm for 15min. After that, for preparation of the prepared real sample in 4.4 μM of MCs, 500 ml of the resulted supernatant was mixed with 1mg of MCs powder (Sigma-Aldrich, USA), and added to 500ml of 0.1M PBS solution containing 1mM ATCl. For the preparation of the real sample of water containing 4.4µM of MCs, the 1mg of MCs powder was injected to 500ml of agricultural wastewater and the resulted sample was added to of 0.1M PBS solution containing 1 mM ATCl in an equal volume ratio.

2.3. Analyses

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were conducted on Autolabpotentiostat/galvanostat system (PGSTAT30, Eco Chemie, Utrecht, Netherlands) using three electrode-electrochemical cells containing a working electrode (unmodified or modified CPE), and a counter electrode (Pt wire) and a reference electrode (Ag/AgCl (KCl 3M). The electrolyte for CV measurements is 0.1M PBS pH 8.0 containing 0.1 M KCl (99%, Sigma-Aldrich, USA) and 4 mM [Fe(CN)₆]³⁻/⁴⁻ (98.5%, Sigma-Aldrich, USA), and electrolyte for DPV measurements is 0.1M PBS pH 8.0 containing 1 mM acetylthiocholine chloride (ATCl, Sigma–Aldrich, USA). 0.1 M PBS WAS prepared from an equal volume ratio of 0.1 M NaH₂PO₄ (99%, Merck, Germany) and 0.1 M Na₂HPO₄ (≥99.0%, Sigma-Aldrich, USA). The morphological analyses of modified electrodes were carried out using scanning electron microscopy (SEM, JSM-5510LV, JEOL Ltd., Tokyo, Japan).

For inhibition measurements, the DPV response of electrode in 0.1M PBS pH 8.0 containing 1 mM ATCl at a scanning rate of 15 mV/s was recorded. Next, the electrode was thoroughly rinsed with deionized water and incubated in an aqueous solution with various concentrations of MCs for 15 minutes, and the inhibition rate was estimated using the resulted DPV responses of the electrode as following equation [24]:

\[
\text{Inhibition rate (\%)} = \frac{I_0 - I_c}{I_0} \times 100
\]  

(1)

Where \(I_0\) and \(I_c\) are the peak current of DPV response of electrode before and after incubation in MCs solution.
3. RESULTS AND DISCUSSION

Figure 1 shows the SEM images of CNTs/CPE and AChE–CS/CNTs/CPE. As observed from Figure 1a, CNTs are homogeneously covered the CPE that forms a net-like film, resulting in the facilitation fixing of the AChE–CS onto the surface of GCE. As seen from Figure 1b, the immobilization of AChE–CS on CNTs/CPE changes the morphology of electrode and a polymer-like connection structure was partially formed among the functionalized CNTs which can be attributed to the wrapping of CNTs with AChE and chitosan chains [25, 26]. After the enzyme immobilization, the tubular structure of CNTs reveals the particle-like morphology which indicated to successful immobilization of AChE–CS on porous structure of CNTs.

![Figure 1. SEM images of (a) CNTs/CPE and (b) AChE–CS/CNTs/CPE.](image)

Figure 2. CV curves of (a) CPE, (b) CNTs/CPE, (c) AChE/CNTs/CPE and (d) AChE–CS/CNTs/CPE in 0.1mM PBS containing 0.1M KCl and 4mM [Fe(CN)₆]³⁻/⁴⁻ at scanning rate of 15mV/s.
The electrochemical properties of CPE, CNTs/CPE, AChE/CNTs/CPE and AChE–
CS/CNTs/CPE were studied by the CV technique in 0.1mM PBS containing 0.1M KCl and 4 mM
[Fe(CN)₆]³⁻/⁴⁻ at a scanning rate of 15mV/s. The obtained CV curves are depicted in Figure 2 that
indicated to the formation of a pair of well-defined redox peaks for all electrodes. Comparison between
the CPE and CNTs/CPE demonstrates that modification the CPE with CNTs significantly enhances the
peak current and CV integral area which implied to enhance surface area, increase in electrochemical
active sites and electron transfer rate due to high aspect ratios, high electrical conductivity and greater
electroactivity of CNTs [27]. The CV curve of AChE/CNTs/CPE from Figure 2c shows the decrease in
peak current because of poor conductivity of AChE bio-macromolecules [28]. Comparison between
the CNTs/CPE and AChE–CS/CNTs/CPE illustrates that the absorption of CS on CNTs/CPE via
covalent link slightly increases the electroactivity and sensitivity of electrode [29]. Moreover, chitosan
is the non-toxic natural hydrophilic polysaccharide with good biocompatibility that makes it excellent
for film-forming and immobilization AChE. Chitosan can increase in electrical conductivity after the
addition to AChE due to its relatively high conductivity [30].

Figure 3. DPV curves of (a) CPE, (b) CNTs/CPE, (c) AChE/CNTs/CPE and (d) AChE–
CS/CNTs/CPE in 0.1M PBS pH 8.0 containing 1mM ATCl at a scanning rate of 50mV/s.

Figure 3 displays the DPV curves of electrodes in 0.1M PBS pH 8.0 containing 1mM ATCl at a
scanning rate of 50 mV/s which indicated the appearance of anodic peak as the electrochemical anodic
peak of CPE,CNTs/CPE, AChE/CNTs/CPE and AChE-CS/CNTs/CPE at the potential of 0.53 V.
There is the higher current for AChE-CS/CNTs/CPE that it can be attributed to Hydrophobic binding
site in AChE which could act as a conductor connecting the substrate to the active site of the enzyme
[31]. Additionally, the proper length of the carbon chain of functionalized CNTs facilitates AChE
expression [32], and porous structure of CNTs provided the more accessible sites for ions diffusion
and charge transfer [33]. High loading capacity and ionic conductivity of chitosan membrane as a
biocompatible polymer enhances the electroactivity and covalently immobilization of AChE on surface
of chitosan [34, 35]. Therefore, further electrochemical studies were conducted on AChE-CS/CNTs/CPE.

Figure 4 shows the results of the study the stability of AChE-CS/CNTs/CPEDPV response in 0.1M PBS pH 8.0 containing 1mM ATCl at a scanning rate of 50mV/s. As observed, the first and 100thDPV responses of AChE-CS/CNTs/CPE indicate to 3% change of reduction peak current. Consequently, AChE-CS/CNTs/CPE shows a very stable response due to the synergistic effect of CNTs, AChE and chitosan. CNTs have been widely used in enzyme immobilization because of their extraordinary properties, such as porous hollow structure, high specific surface area, large aspect ratios, and super hydrophobic surface [36]. Chemical modification of surface of CNTs through the acid treatment and formation of the polar groups could improve the activity of AChE [37]. Chitosan and functionalized CNTs can create a stable complex through noncovalent binding which probably can promote the electrochemical performance and stability of the sensor [38]. Studies also have been shown that chitosan with strong absorptive ability and subtle electronic properties has high biocompatibility for acetylacetonate complexes [39, 40].

![Figure 4](image)

**Figure 4.** (a) First and (b) 100th DPV responses of AChE-CS/CNTs/CPE in 0.1M PBS pH 8.0 containing 1mM ATCl at a scanning rate of 50mV/s.

Figure 5 shows the DPV responses of AChE-CS/CNTs/CPE before and after the addition of 20, 40 and 60µM MCs in 0.1M PBS pH 8.0 containing 1mM ATCl at a scanning rate of 50mV/s. As seen, and the decrease in peak current increased with the increasing concentration of MCs the peak current is decreased which related to the irreversible inhibition action of MCs on AChE and reduction of the enzymatic activity of AChE in the substrate [41]. Therefore, the MCs concentration can be detected through the change in peak current of the AChE-CS/CNTs/CPE [42]. Moreover, the chitosan modified
the CNTs/CPE can increase the adsorption to AChE and enhance the sensitivity of AChE-CS/CNTs/CPE.

Figure 5. The DPV responses of AChE–CS/CNTs/CPE (a) before and after additions of (b) 20, (c) 40 and (d) 60µM MCs in 0.1M PBS pH 8.0 containing 1mM ATCl at a scanning rate of 50mV/s.

The period of incubation for the inhibition of pesticide is an important parameter in electrochemical analyses. In such a way that the inhibition of AChE is increased with increasing incubation time [43, 44]. The required incubation period for the inhibition was investigated at various time intervals (5 to 60 minutes). Figure 6 shows that the inhibition is drastically increased with increasing incubation time, and reached its maximum at around 15 minutes in 0.04µM MCs solution. Therefore, 10 minutes is used as the optimal incubation time for the electrochemical analyses.

Figure 6. Results of study of inhibition time on the response of the AChE-CS/CNTs/CPE in 0.1M PBS pH 8.0 containing 1 mM ATCl for inhibition of 0.04µM MCs
Figure 7 presents the DPV measurement and obtained calibration plot of AChE-CS/CNTs/CPE in 0.1M PBS pH 8.0 containing 1mM ATCl for successive additions of 10µM MCs at a scanning rate of 50 mV/s. The sensitivity, linear range and limit of detection of sensor are estimated 0.9038µA/µM, 1 to 70µM and 0.3nM, respectively. Table 1 shows the comparison of sensing results of AChE-CS/CNTs/CPE with the other reported MCs sensor that it reveals the acceptable performance of MCs sensor of this study. Furthermore, AChE-CS/CNTs/CPE exhibit a broad linear range for determination MCs that can be attributed to a combination of chitosan polymer and AChE, and high porous and more defect on functionalized CNTs as a nanostructured substrate which provides a great many electroactive sites on AChE-CS/CNTs/CPE.

Figure 7. (a) The a DPV measurement and (b) obtained calibration plot of AChE–CS/CNTs/CPE in 0.1M PBS pH 8.0 containing 1mM ATCl for successive additions of 10µM MCs at a scanning rate of 50 mV/s.

Table 1. Comparison of sensing results of AChE–CS/CNTs/CPE with the other reported MCs sensor.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Technique</th>
<th>Linear Range (µM)</th>
<th>limit of detection (nM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE–CS/CNTs/CPE</td>
<td>DPV</td>
<td>1 to 84</td>
<td>0.3</td>
<td>This work</td>
</tr>
<tr>
<td>Cl/MWCNTs/horseradish peroxidase/AChE/GCE</td>
<td>DPV</td>
<td>10^{-5} to 10^{-1}</td>
<td>0.0045</td>
<td>[28]</td>
</tr>
<tr>
<td>Cl/iron porphyrin/MWCNTs/AChE/GCE</td>
<td>DPV</td>
<td>10^{-4} to 10^{-1}</td>
<td>0.032</td>
<td>[45]</td>
</tr>
<tr>
<td>AChE/silica sol–gel assembling Au NPs</td>
<td>Amperometry</td>
<td>9 to 67.2</td>
<td>1.3</td>
<td>[46]</td>
</tr>
<tr>
<td>AChE/MWCNTs/Au NPs-chitosan/GCE</td>
<td>FFTCCV*</td>
<td>0.1 to 10</td>
<td>10</td>
<td>[47]</td>
</tr>
<tr>
<td>AChE/cysteamine/Au NPs/GCE</td>
<td>CV</td>
<td>0.05 to 2.7</td>
<td>2.7</td>
<td>[48]</td>
</tr>
<tr>
<td>AChE/ZnO/Pt</td>
<td>CV</td>
<td>-</td>
<td>0.036</td>
<td>[49]</td>
</tr>
<tr>
<td>AChE/CdTe QDs–GNPs composite</td>
<td>CV</td>
<td>0.004 to 4.5</td>
<td>1.3</td>
<td>[50]</td>
</tr>
</tbody>
</table>

* FFTCCV: Fast fourier transform continuous cyclic voltammetry
Further DPV measurements were carried out for the study of selectivity and interference effect of AChE-CS/CNTs/CPE as MCs sensor. Table 2 shows the resulted DPV currents of AChE-CS/CNTs/CPE in 0.1M PBS containing 1mM ATCl at a scanning rate of 50mV/s for additions of 1µM MCs and 10µM of the common foreign species and possible interfering compounds of pesticides. Results show that the change of DPV current for additions of MCs is significantly higher than those other substances which implied anti-interference ability and the selective response of proposed electrode to determination of MCs.

Table 2. The resulted DPV measurements of AChE–CS/CNTs/CPE in 0.1M PBS containing 1mM ATCl at a scanning rate of 50mV/s for additions of 1µM MCs and 10µM of the common foreign species and possible interfering compounds of pesticides.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Concentration (µM)</th>
<th>Change of DPV current (µA)</th>
<th>RSD* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCs</td>
<td>1</td>
<td>0.9028</td>
<td>±0.0088</td>
</tr>
<tr>
<td>methyl parathion</td>
<td>10</td>
<td>0.0710</td>
<td>±0.0019</td>
</tr>
<tr>
<td>ethyl parathion</td>
<td>10</td>
<td>0.0101</td>
<td>±0.0011</td>
</tr>
<tr>
<td>nitrophenol</td>
<td>10</td>
<td>0.0089</td>
<td>±0.0107</td>
</tr>
<tr>
<td>quinolphos</td>
<td>10</td>
<td>0.0601</td>
<td>±0.0041</td>
</tr>
<tr>
<td>pirimicarb</td>
<td>10</td>
<td>0.0098</td>
<td>±0.0006</td>
</tr>
<tr>
<td>isoprocarb</td>
<td>10</td>
<td>0.0107</td>
<td>±0.0005</td>
</tr>
<tr>
<td>methiocarb</td>
<td>10</td>
<td>0.0095</td>
<td>±0.0004</td>
</tr>
<tr>
<td>phorate</td>
<td>10</td>
<td>0.0316</td>
<td>±0.0045</td>
</tr>
<tr>
<td>methomyl</td>
<td>10</td>
<td>0.0215</td>
<td>±0.0016</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>10</td>
<td>0.0181</td>
<td>±0.0011</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>10</td>
<td>0.0201</td>
<td>±0.0010</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>10</td>
<td>0.0319</td>
<td>±0.0013</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>10</td>
<td>0.0411</td>
<td>±0.0012</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>10</td>
<td>0.0098</td>
<td>±0.0006</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>10</td>
<td>0.0227</td>
<td>±0.0013</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>10</td>
<td>0.0195</td>
<td>±0.0004</td>
</tr>
</tbody>
</table>

* RSD: relative standard deviation

Figure 8. (a) The DPV measurements and (b) related calibrations plots of AChE-CS/CNTs/CPE in 0.1M PBS pH 8.0 containing 1mM ATCl for successive additions of 10µM MCs at a scanning rate of 50mV/s for prepared strawberry.
Figure 9. (a) The DPV measurements and (b) related calibrations plots of AChE-CS/CNTs/CPE in 0.1M PBS containing 1mM ATCl for successive additions of 10µM MCs at a scanning rate of 50 mV/s for prepared agricultural waste water samples.

Table 3. The obtained analytical results for determination of MCs in prepared strawberry and agricultural waste water samples by the standard addition method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>added (µM)</th>
<th>Found (µM)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>strawberry</td>
<td>10.00</td>
<td>9.96</td>
<td>99.60</td>
<td>2.67</td>
</tr>
<tr>
<td></td>
<td>20.00</td>
<td>19.90</td>
<td>99.50</td>
<td>2.89</td>
</tr>
<tr>
<td></td>
<td>30.00</td>
<td>29.89</td>
<td>99.63</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>40.00</td>
<td>39.91</td>
<td>99.77</td>
<td>4.06</td>
</tr>
<tr>
<td>agricultural</td>
<td>10.00</td>
<td>9.87</td>
<td>98.70</td>
<td>1.77</td>
</tr>
<tr>
<td>waste water</td>
<td>20.00</td>
<td>19.78</td>
<td>98.90</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td>30.00</td>
<td>29.91</td>
<td>99.70</td>
<td>3.38</td>
</tr>
<tr>
<td></td>
<td>40.00</td>
<td>39.84</td>
<td>99.60</td>
<td>4.08</td>
</tr>
</tbody>
</table>

Practical application of AChE-CS/CNTs/CPE to the determination of MCs in prepared real samples of strawberry and agricultural wastewater was investigated though DPV technique in 0.1M PBS containing 1mM ATCl for successive additions of 10µM MCs at a scanning rate of 50 mV/s. Figures 8 and 9 show the DPV measurements and related calibrations plots for prepared strawberry and agricultural wastewater samples, respectively. As observed from the calibration plots in Figures 8 and 9, the MCs content in prepared strawberry and agricultural wastewater samples are 4.28µM and 4.37µM, respectively which value very close to the initial concentration of injected MCs solution in the preparation process of real samples. In addition, the obtained recovery and RSD values in Table 3 by the standard addition method shows the acceptable values (98.70 to 99.77% for recovery and 1.77 to 4.08% for RSD) and indicates to good accuracy of AChE-CS/CNTs/CPE for MCs detection in fruits and water samples.
4. CONCLUSION

This study was carried out for synthesis and electrochemical studies of AChE-CS/CNTs/CPE for the determination of MCs in water and fruits samples. For the preparation of the modified electrode, the functionalized CNTs were modified the CPE surface, and then AChE was immobilized through the mixture of AChE-CS on the CNTs/CPE surface. Results of the morphological analysis showed that CNTs were homogeneously covered the CPE that forms a net-like film, resulting in the facilitation fixing of the AChE-CS onto the surface of GCE. The result of electrochemical studies indicated that the AChE-CS/CNTs/CPE can act as high stable, wide linear range and selective MCs sensor. The sensitivity, linear range and limit of detection of sensor were estimated at 0.9038µA/µM, 1 to 70µM and 0.3nM, respectively. The practical application of AChE-CS/CNTs/CPE to the determination of MCs in prepared real samples of strawberry and agricultural wastewater was investigated and analytical results showed the acceptable values for recovery and RSD which indicated to good accuracy of the proposed method for MCs detection in fruits and water samples.

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