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Analytical Methods

# A naphthoquinone/SAM-mediated biosensor for olive oil polyphenol content

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ABSTRACT

We report on the design of an amperometric tyrosinase-based biosensor using a self-assembled monolayer of ω-mercaptopropyl naphthoquinone on gold electrode as an electron mediator. Under optimal conditions (*i.e.* pH = 7.4 and E = −0.35 V vs. KCl), the chronoamperometric response of the naphthoquinone-modified bioelectrode to successive additions of phenol was evaluated. The biosensor exhibits sensitive bioelectrocatalytic response at a working potential of −0.35 V vs. Ag/AgCl (sat.KCl), reaching the steady-state current within 40 s after each addition of phenol solution with a range of 0–135 μM and a limit of detection and quantification which are 0.019 μM and 0.0633 μM, respectively. The bioelectrode was used to determine the content in polyphenol in a local virgin olive oil.



## 1. Introduction

Tunisia ranks among the major olive oil producers. In fact, in 2015, Tunisia was the world's second largest olive oil producer after Spain. Olive oil is considered as a fundamental component of Mediterranean diet, which has been associated with a lower incidence of coronary heart disease. Furthermore, it is widely used in cooking and in the storage of vegetable and animal food (Willett et al., 1995). It provides a rich source of phenolic compounds, including simple phenols, lignans, and secoiridoids (Owen et al., 2000). Olive oil has a beneficial effect on human health attributed to its content in phenolic compounds, which continue to become increasingly important because of their health virtues. Phenolic derivatives are also used as additives for food industry, pharmaceutical and also cosmetics (Assimopoulou, Zlatanos, & Papageorgiou, 2005). Therefore, analytical determination of phenolic compounds is an important parameter to be considered when evaluating the virgin olive oil quality. Several methods are available including gas or liquid chromatography and spectrophotometry (Chriswell et al., 1975; Poerschmann, Zhang, Kopinke, & Pawliszyn, 1997). However, some of these techniques are time-consuming, complex to perform and expensive, which limits their application for rapid and real-time detection (Han et al., 2015).

Electrochemical biosensors for the analysis of phenols with laccase as biological recognition element, which is known to reduce oxygen directly to water without the intermediate formation of hydrogen peroxide (Torrecilla, Mena, Yáñez-Sedeño, & García, 2007), have been well developed (Rodríguez-Delgado et al., 2015) by the immobilization of laccase on different electrode surfaces such as glassy carbon (Freire, Thongngamdee, Durán, Wang, & Kubota, 2002), graphite (Kulys & Vidziunaite, 2003) and gold surface (Vianello et al., 2004). Furthermore, amperometric biosensors based on tyrosinase as the biological element has been shown to be a very simple and convenient tools for phenol assay due to their high sensitivity, effectiveness, and simplicity (Dempsey, Diamond, & Collier, 2004; Liu, Yan, Zhu, Zhang, & Liu, 2015; Yu, Liu, & Ju, 2003; Tatsuma & Sato, 2004; Han et al., 2015; Haddaoui & Raouafi, 2015). These devices also allow rapid screening of phenol levels and can be miniaturized for on-site analysis.

One of the most important factors during these biosensors' elaboration is the effectiveness of the enzyme immobilization on electrode surface, which usually yields in high analytical performances. In fact, the electron-transfer can be greatly affected which hinder the bioactivity and stability of the biorecognition molecules entrapped inside (Liu, Feng, Zhao, Wang, & Liu, 2012; Song, Li, Li, Li, & Long, 2011; Wang, Tan, Zhao, & Liu, 2008). As far as we know,

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there is scarce reports on amperometric biosensors for the determination of content of phenols in olive oil (Capannesi, Palchetti, Mascini, & Parenti, 2000; Torrecilla et al., 2007). This work current is a contribution in this field, we report on the design of a biosensing device, which is simple to build, amenable and cost-effective. We used electrochemical methods to study the performances of the bioelectrode. The latter was applied to determine the concentration of phenols in olive oil.

## 2. Experimental sections

### 2.1. Apparatus and measurements

Electrochemical experiments: cyclic voltammetry (CV), chronoamperometry (CA) and electrochemical impedance spectroscopy (EIS) were carried out on a Metrohm Autolab PC-controlled PGstat M204. A conventional three-electrode cell was used for all the electrochemical experiments, which was fitted with a platinum wire as an auxiliary electrode, an Ag/AgCl (sat.KCl) as a reference electrode, and a modified gold electrode (3-mm diameter, Metrohm, Switzerland) as a working electrode. All electrochemical measurements were taken at room temperature (RT). For EIS measurements, the bare and modified electrodes were immersed into a 0.1 M phosphate-buffered solution (PBS) containing a mixture of 5 mM concentrations of $[Fe(CN)_6]^{3-/4-}$ as a redox probe and the frequency was swept from 100 kHz to 0.1 Hz at an applied potential of 200 mV with an amplitude modulation of ±20 mV.

For X-ray photoelectron spectroscopy (XPS) signals, they were recorded on gold electrode chip using a Thermo Scientific K-Alpha XPS system (Thermo Fisher Scientific, UK) equipped with a micro-focused, monochromatic Al Kα X-ray source (1486.6 eV). The spectra were acquired in the constant analyzer energy mode with pass energy of 200 eV for the survey. Narrow regions were collected with pass energy of 50 eV. Charge compensation was achieved with the system flood gun that provides low energy electrons (~0 eV) and low energy argon ions (20 eV) from a single source. The argon partial pressure was $3 \times 10^{-7}$ mbar in the analysis chamber. The Thermo Scientific Avantage software, version 5.943 (Thermo Fisher Scientific), was used for digital acquisition and data processing. Spectral calibration was determined by using the automated calibration routine and the internal Au, Ag and Cu standards supplied with the K-Alpha system. The surface compositions (in atomic %) were determined by considering the integrated peak areas of detected atoms and the respective sensitivity factors.

### 2.2. Reagents and stock solutions

All chemicals were analytical reagent grade. 1,4-naphthoquinone (98%), propan-1,3-dithiol (98%), tyrosinase from mushroom (EC 1.14.18.1, lyophilized powder, 1000 unit/mg), chitosan (CS), phenol (99.5%), PBS tablets (98%), tetrabutylammonium tetrafluoroborate ($TBABF_4$, 99%) were purchased from Sigma–Aldrich. Buffer and phenol solutions were prepared using deionized water (Milli-Q Millipore system). A daily fresh stock solution of phenol at c = 0.1 M in PBS was used to prepare, by dilution, the daughter solutions which were used in the experiments. The ω-mercaptopropyl naphthoquinone was prepared according to literature (Hammami, Sahli, & Raouafi, 2016; Katritzky, Fedoseyenko, Mohapatra, & Steel, 2008).

### 2.3. Biosensor preparation

Gold electrodes were first mechanically polished with aqueous slurries of fine alumina powders (0.1 and 0.05 μm) on a polishing cloth then successively sonicated in acetone, water and ethanol, each during 3 min. The electrodes were further electrochemically polished in 0.5 M sulfuric acid by applying 3 cyclic voltammetric scans between −0.1 and 1.5 V at 0.05 $Vs^{-1}$. The electrode was then immersed into 1.0 mM dichloromethane solution of the ω-mercaptopropyl naphthoquinone overnight. The electrode is then washed with deionized water. 5 μL of tyrosinase solution (1 mg per 100 μL) in phosphate buffer pH 7.4 containing 2.5% chitosan was then deposed on the gold electrode surface and allowed to dry at RT for 60 min. The electrode was then washed with ultrapure water, dried by a nitrogen flow and used immediately for the biosensing. When it is not used, the electrode is stored at 4 °C.

### 2.4. Olive samples

Liquid–liquid extraction was used to extract the phenolic compounds from olive oil. The optimal extraction conditions used were from the study of Carrasco Pancorbo et al. (2004). 60 g of oil were added to 60 mL of hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v) solution. The combined extracts of the hydrophilic layer were dried in a rotary evaporator under reduced pressure and a temperature of 40 °C. Finally, the residue was re-dissolved in 0.5 mL of methanol/water (50:50, v/v) and filtered.

## 3. Results and discussion

### 3.1. Structural characterization

Quinone derivatives are typical compounds used for the study of proton-coupled electron-transfer; they usually exchange simultaneously two protons and two electrons (Zhang, Rosendahl, & Burgess, 2010). Fig. 1a depicts the cyclic voltammograms of ω-mercaptopropyl naphthoquinone assembled on gold electrode (NQ-SAM/GE) as function of the sweep rate. In this case, the ratio of anodic-to-cathodic currents ($i_{pc}$-to-$i_{pa}$) is less than the unity and the peak-to-peak potential separation is larger than theoretical value of 0 mV expected for the transfer of two electrons indicating that the electron-transfer is slow. Plotting the current versus the scan rate gives a straight line, which implies that we are in presence of an adsorption-controlled electrochemical process (Inset in Fig.1).

Chitosan is a nontoxic biopolymer with a large amount of positive charges, which is widely used for enzyme immobilization to develop biosensors due to its special properties, including excellent film-forming ability and good biocompatibility (Zhang, Li, Wu, Jiang, & Hu, 2014). In our case, without chitosan addition, the enzyme cannot be firmly immobilized on SAM-modified gold electrode. No noticeable changes were detected on the cyclic voltammograms recorded before and after the enzyme addition. When a mixture of tyrosinase/chitosan is casted on the electrode surface, as displayed in Fig. S1, the electrochemical features of the electrode become more reversible which in favor of the successful tyrosinase immobilization (Fig. S1).

To study the surface features of the enzyme-modified electrodes, we used electrochemical impedance spectroscopy. Fig. 2 displays the Nyquist plots of the bare gold electrode, and NQ-SAM-and tyrosinase-modified (Tyr/CS/NQ-SAM-) electrodes. The semicircle diameter provides useful information on the electron-transfer resistance, which depicts the electron-transfer kinetics of redox probe at the electrode interface usually observable at higher frequencies. The electron-transfer resistance of the Tyr/CS/NQ-SAM/GE was the largest one showing that addition of chitosan to tyrosinase provokes hindrance of the macromolecular structure of enzyme toward the electron-transfer.

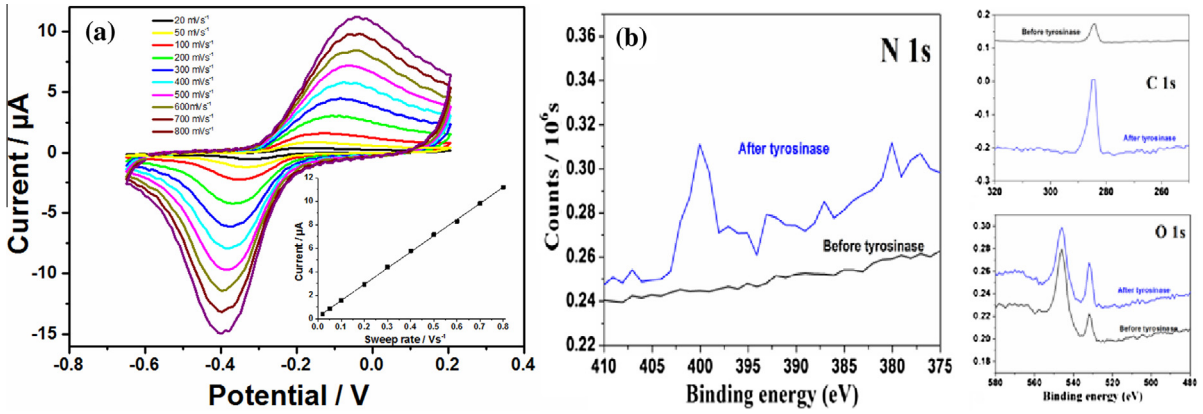


**Fig. 1.** a) CVs of NQ-SAM/GE according to scan rates vs Ag/AgCl as a reference electrode (pH = 7.0, 0.1 M phosphate buffer). Inset: plot of current vs. scan rate. b) XPS spectra of NQ-SAM before and after reacting with tyrosinase, peaks corresponding to N1s, C1s and O1s.

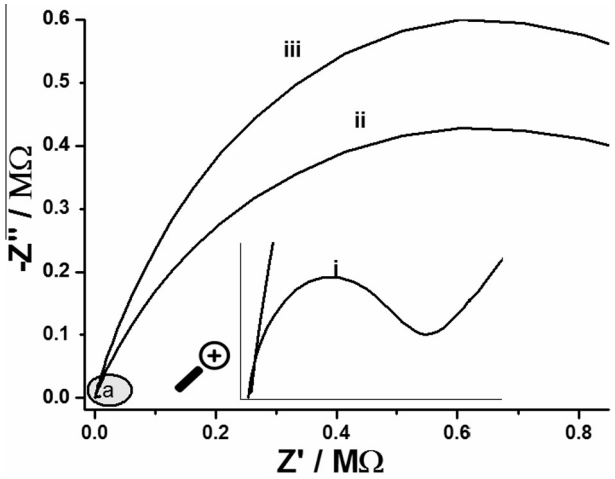


**Fig. 2.** EIS plots at frequency range from 0.1 Hz to 100 kHz of (i) bare gold electrode, (ii) NQ-SAM and (iii) Tyr/CS/NQ-SAM/GE in 5 mM of $[Fe(CN)_6]^{3/4-}$ solution. pH = 7.0.

In addition, XPS analysis which provide important information regarding the chemical transformation of molecules adsorbed to solid surface was conducted on the NQ-SAM electrode before and after addition of enzyme (Fig. 1b). After addition of tyrosinase, a new peak is observed in the N1s region at 400 eV, implying the presence of nitrogen belonging to the enzyme. In addition, presence of enzyme provoke increase of C1s signal at 284.5 eV characteristic of the alkyl part consistent with the fact that enzyme is immobilized to solid surface.

### 3.2. Analytical performances of the biosensor

Fig. 3a shows the cyclic voltammetric behavior of tyrosinase-modified electrode in the presence and absence of phenol. As it can be seen, the presence of phenol induces an important increase of the reduction current denoting a catalytic effect. This current is much higher than in the case of enzyme absence, when only a very small current increase was registered denoting the beneficial effect of introducing the enzyme (Fig. 3b). We think that the enzyme transforms the phenol into catechol then into o-quinone, which accumulates on the electrode surface. The concomitant reduction of the self-assembled naphthoquinone and o-quinone generated higher current.

The chronoamperometric response of the Tyr/CS/NQ-SAM/GE biosensor upon successive additions of phenol was further evaluated. The biosensor shows a sensitive bioelectrocatalytic response, reaching a steady-state current within 40 s after each phenol addition (Fig. 4a). The calibration curve of the biosensor is shown in Fig. 4b depicting a linear response within the range from 0.02 to 135 µM phenol ($R^2 = 0.9989$) and a sensitivity of 0.0203 µA $\mu M^{-1}$.

Moreover, the limits of detection and quantification are respectively 0.019 µM and 0.0633 µM calculated according to the 3.3 times *s*/*m* and 10 times *s*/*m* criteria, respectively; where *s* stands for the standard deviation of the peak current of low concentrations of the analyte (five runs) and *m* is the slope of the related calibration graph (Mayorga-Martinez et al., 2014).

The analytical performances of different configurations of tyrosinase-based biosensors are gathered in Table 1. It can be noticed that both limit of detection and dynamic range of the prepared biosensor are better than those of Mayorga-Martinez, Hlavata, Miserere, López-Marzo, Labuda, Pons and Merkoçi (2013), Mayorga-Martinez et al. (2014) and from Haddaoui and Raouafi (2015). Furthermore, working potential is low (−0.35 V vs Ag/AgCl), which permits to avoid undesired currents arising from the interfering compounds.

### 3.3. Selectivity and stability of the biosensor

The biosensor response was tested against the interferences of structurally related analytes to phenol such as aniline, benzaldehyde or ascorbic acid. These species are usually present with phenolic compounds in contaminated samples (Mayorga-Martinez et al., 2013). The concentrations of substrates were taken 10-folds higher than that of phenol. As shown in Fig. S2, none of the tested substrates produces any measurable perturbation of the steady-state current except ascorbic acid. When the Tyr/CS/NQ-SAM/GE biosensor is stored at 4 °C for a period of 2 weeks, no remarkable decrease in the biosensor response of phenol is observable. After 3 weeks, the biosensor retains more 75% of its activity. Fig. S3 depicts the temporal stability as function of the number of weeks.

### 3.4. Phenol detection in virgin olive oil

To evaluate the applicability of the proposed method in real samples, we used it to determine phenol levels in virgin olive oil. The pretreatment process was performed as detailed in the experimental section. The extract was diluted 10 times in order to avoid matrix effects. From the current recorded for the extract, we esti-

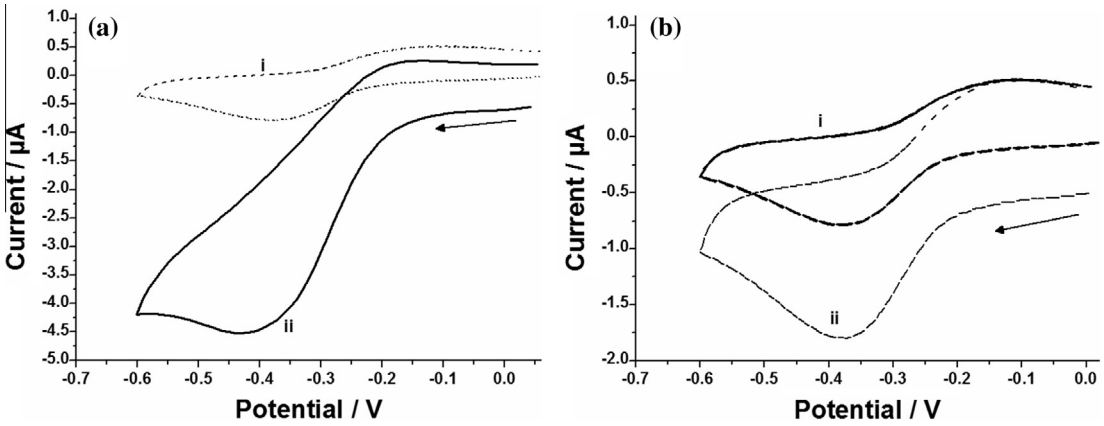


**Fig. 3.** CVs of Tyr/CS/NQ-SAM/GE (pH = 7.4) at 50 mV $s^{-1}$ in presence (a) and absence (b) of tyrosinase, (i) before and (ii) after addition of 0.1 mM of phenol; 0.1 M phosphate buffer, Scan rate 50 mV $s^{-1}$. Arrow indicates the scanning direction.

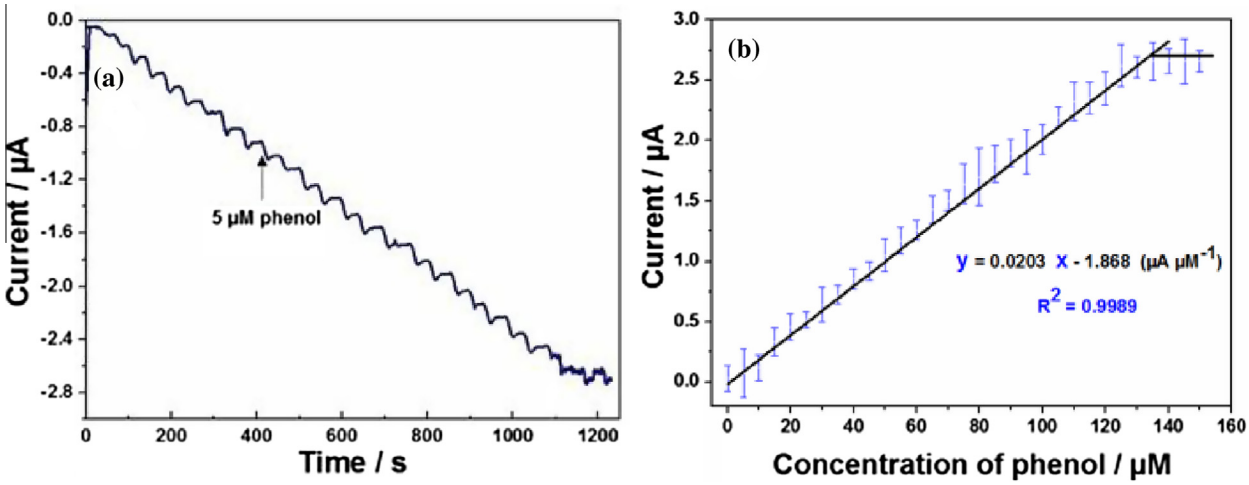


**Fig. 4.** a) Current–time response of the Tyr/CS/NQ-SAM/GE for the successive additions of 5 µM phenol solution, under stirring at a working potential of −0.35 V vs. Ag/AgCl (sat.KCl) at 0.1 M phosphate-buffered solution (pH = 7.4). b) Biosensor calibration curve displaying current versus phenol concentrations.

**Table 1**
Comparison of present work with other published electrodes for phenols determination.

| Electrode material[1] | Analytes | Linear range (µM) | Detection limit (µM) | Potential (V) | Ref. |
|---|---|---|---|---|---|
| SPCE/IrOxNPs/GA/Tyr | Catechol | 0.25–27.5 | 0.080 | −0.2 V vs. Ag/AgCl | Mayorga-Martinez et al. (2013, 2014) |
| SPCE/ZnONPs/GA/Tyr | Phenol | 0.1–14 | 0.020 | −0.2 V vs. Ag/AgCl | Haddaoui and Raouafi (2015) |
| Microfluidic/SPCE/$CaCO_3$/PEI-MPs/GA/Tyr | Phenol | 0.5–5 | 0.030 | −0.2 V vs. Ag/Ag Cl | Mayorga-Martinez et al. (2013) |
| QDs/Chit/Tyr | Phenol | 650–3000 | 0.001 | −0.2 V vs. ECS | Han et al. (2015) |
| NQ/Chit/Tyr/Au | Phenol | 0–135 | 0.019 | −0.35 V vs. Ag/AgCl | Present work |

[1] SPCE: screen-printed carbon electrode; IrOx: iridium oxide; GA: glutaraldehyde; Tyr: tyrosinase; Bi: bismuth; Chit: chitosan; NPG: nanographene; PEI: poly(ethyleneimine), MPs: microparticles. QDs: quantum dots.

mated the phenol levels in the oil samples, expressed as µM of phenol to 63.56 (Fig. S4). This value is slightly larger of that of Spanish (A) and Italian (B) olive oils which were 58.0 µM and 35.5 µM, respectively (Wang, Reviejo, & Mannino, 1992).

Additionally, accuracy of the developed method and the matrix interferences were evaluated. Analytical recovery experiments were performed by adding known amounts of phenol into two samples of local olive oils. The percentages of the recovery values were calculated by comparing the concentration obtained from the samples with actual and added concentrations. It is observed that there is almost no influence of the matrix on the proposed biosensor. The R.S.D (n = 4) was less than 2.5%, indicating that this method can be applied for an available electrochemical sensor of phenols Virgin olive oils (Table 2).

## 4. Conclusion

In this work, we developed a simple and convenient method to prepare a phenol biosensor by immobilizing tyrosinase into a chi-

**Table 2**
Results of virgin olive oil recovery using Tyr/CS/NQ-SAM/GE as working electrode.

| Samples[1] | Diluted samples (μM)[a] | Added (μM) | Found (μM) | RSD (%) | Recovery (%)[b] |
|---|---|---|---|---|---|
| 1 | 6.430 | 50 | 56.449 | 1.4 | 100.03 |
| 2 | 6.628 | 50 | 56.484 | 2.2 | 99.74 |

[a] Represents the average of four times determination concentration. Dilution is 10 folders.
[b] Recovery (%) = concentration of the analyte after adding a known amount of standard to the real sample/(quantified analyte concentration in the real sample + the concentration of a known amount of standard that was spiked in the real sample) * 100.
[1] V.O.O: virgin olive oil.

tosan matrix on gold electrode surface modified with a self-assembled quinoid compound. It provides a selective and sensitive biosensor for phenol at a low working potential −0.35 V vs. Ag/AgCl (sat.KCl). The designed biosensor is applied to the determination of phenol levels in virgin olive oil samples with satisfactory results. The method is simple and can be easily used the electrochemical biosensing of phenol levels in olive oils.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.073.

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