Full Paper

Development of an Electroanalytical Method for the Quantification of Zearalenone (ZEA) in Maize Samples

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Abstract
The application of electroanalytical techniques to detect and quantify zearalenone (ZEA) mycotoxin that frequently contaminates maize and foodstuff is studied in this work. Rice and maize grains were inoculated with *Fusarium* fungus to obtain ZEA in artificially infected samples. The electro-oxidation of ZEA adsorbed on the surface of glassy carbon (GC) electrodes in 20% acetonitrile (ACN) + 80% 1 M $HClO_4$ (aqueous solution) reaction medium was studied by using square-wave voltammetry (SWV). Studies were conducted to find the most favorable accumulation potential (E_{acc}) and accumulation time (t_{acc}) to perform the ZEA preconcentration on the electrode surface. It was found that E_{acc} was any value in the range from 0.00 – 0.90 V and the best t_{acc} was 120 s, respectively, for ZEA separated from extracting solution by TLC (ZEA_{TLC}) while $E_{acc} = 0.90$ V corresponded to ZEA in non separated matrix solution (ZEA_{matrix}). The ZEA quantitative determination was performed by SWV combined with the standard addition method. Linear plots were obtained from the net peak current ($I_{p,n}$) vs c^*_{ZEA} in the concentration range from 20 to 3184 ppb. Detection limit of 30 ppb at a signal to noise ratio of 3:1 was obtained. On the other hand, recovery experiments were performed on uncontaminated maize samples spiked with ZEA.

Keywords: Zearalenona (ZEA), Mycotoxins, Maize, Square-wave voltammetry, Adsorptive accumulation

1. Introduction

Zearalenone (MW = 318.4 g mol^{-1}), whose chemical structure is shown in Figure 1, is a secondary fungal metabolite produced by several species of *Fusarium*, mainly by *Fusarium graminearum* and *Fusarium culmorum* [1]. Since several notations for zearalenona have been used in the literature (ZEA, ZEN, ZON) by different authors [2 – 5], we decided to use ZEA, which is apparently the most widely used.

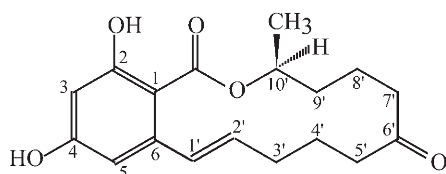

Fig. 1. Chemical structure of Zearalenone (MW = 318.4 g mol^{-1})

Storage of *Fusarium*-infected cereals under wet conditions can result in high levels of ZEA, being maize one of the most frequently contaminated commodity. In addition, ZEA production has been reported on grains in the field during harvest commercial grain processing and/or during storage of any food or feedstuff containing the grain [1]. It has been reported that ingestion of mycotoxins of the *Fusarium* genus via contaminated cereals may lead to fertility disturbance and other reproductive pathologies [2]. Within the certification process of a reference material for the determination of ZEA in maize, short and long-time stability tests of naturally contaminated maize have been performed [3]. Chromatographic methods have been the ones most frequently used for analysis of ZEA and ZEA derivates [2, 4 – 8] while immunoassays methods and UV-vis absorption techniques have also been used for ZEA analysis [4 – 6]. On the other hand, electroanalytical chemistry of mycotoxins is a new field under development. As far as we know, Visconti et al. published the first paper dealing with electrochemical data of some mycotoxins, which were used for chromatographic analysis in the early nineties [9]. Since then, most of studies about the electrochemical behaviour of several mycotoxins produced by a variety of fungi, particularly *Alternaria alternata*, *Cercospora* and *Fusarium*, have been undertaken in our laboratory. Electrochemical data for alternariol (AOH), alternariol mono-methyl ether (AME) and altertoxin I (ATX-I), produced by *Alternaria* [10 – 12], were obtained in different reaction media by using cyclic (CV), differential pulse (DPV) and square-wave (SWV) voltammetries [13, 14]. Besides, important improvements in electrochemical signal and also in detection limit are obtained when the discharge of AME is performed on self-assembled monolayers of dodecanethiol on gold electrodes [15]. Through studies performed on graphitic carbon based bioelectrodes, it has been shown that AME and AOH are substrates of the tirosynase enzyme [16]. Very recently, it has also been demonstrated that these mycotoxins are also

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substrates of peroxidases obtained from turnip [17]. It has also been found that altenueno (ALT), zearalanone (ZAN) and cercosporin (CER) can be discharged electrochemically under proper conditions, opening the possibility for performing mechanistic studies and the implementation of electroanalytical techniques for mycotoxins determination and quantification [18–20]. The discovery of adsorptive properties for ZAN, CER and ATX-I on carbon electrodes [19, 21, 22], has led us to the development of adsorptive stripping techniques [23] with detection limits in the ppb order.

Due to the very important implications of ZEA presence in human beings and animal health as well as from economical aspects, it is necessary to have rapid and economic techniques for its quantification [24]. In this paper, we propose an alternative method that satisfies the previous requirements. Thus, we report studies about the use of SWV on ZEA preconcentrated on the surface of glassy carbon (GC) electrodes combined with the standard addition method [25] for the detection and quantification of ZEA in real samples. The method has been applied to cultures of *Fusarium graminearum* on both rice and maize grains either on the extracting solution itself or after separation procedures from complex matrix. Recovery experiments were also performed on maize samples free of toxin spiked with known amounts of ZEA.

2. Experimental

2.1. Reagents

Commercial ZEA was obtained from Sigma Chemical Company and was used as received. Acetonitrile (ACN), acetone, benzene, chloroform, methanol and hexane were Sintorgan (HPLC grade). Water was from the Labconco waterPro Mobile System Model 90901-01 (HPLC grade water). Perchloric acid and NaCl (Merck p.a) were used as received. Rice and maize grains were from a local commercial store.

Manipulation of all laboratory material was done using plastic gloves for safety reasons.

2.2. Sources of Contaminated Samples

Fusarium graminearum strain (Z-3636) was cultured on both rice and maize in order to have artificially infected samples. Samples of 25 g were brought to about 0.995 water activity in a 250 mL Erlenmeyer flask, autoclaved for 30 min at 121 °C before fungal inoculation and then incubated in the dark for 4 weeks at 25 ± 1 °C [26].

2.3. Toxin Extraction

The ZEA generated after incubation period in samples of both maize and rice was extracted using the AOAC method [27]. Twentyfive g of each sample was placed in a 1000 mL Erlenmeyer flask containing 125 mL methanol-water (60:40), 80 mL hexane and 2 g of NaCl.

The mixture was shaken during 30 min in an oscillating shaker and then filtered. Two extractions with chloroform were performed on 25 mL of the filtered extract. The chloroform phase was dried using a rotary evaporator. Extract solutions were then prepared in benzene. They were stored at 5 °C in the dark. For detection by TLC, the final residue after benzene evaporation was dissolved in 0.20 mL benzene-ACN (98:2). Chromatograms demonstrated that other compounds were also present in the residue. The same procedure was performed on maize samples free of ZEA to obtain an uncontaminated substrate usable for recoveries studies (see below). The pure ZEA was obtained from either rice or maize samples by separation through thin layer chromatography using TLC silica 60 G plates (Merck, Darmstadt), filtered and dissolved in ACN.

The residue of the extract was dissolved in 0.20 mL of ACN for electrochemical detection. From this extract solution, stock solutions of a concentration at least ten times smaller than the extract solution were prepared by dilution. Working solutions were prepared daily by adding aliquots of the stock solution to the 20% ACN + 80% $HClO_4$ (aqueous solution) reaction medium.

2.4. Mycotoxins Analysis

The purity of the standards was determined by TLC [27] and UV-vis absorption spectra [28].

The extracts were screened for ZEA by spotting 10 μL extract together with standards on precoated silica-gel plates (layer thickness 0.25 mm) and using chloroform-acetone (90:10) as the developing solvent. After running, chromatograms were dried and observed under 254 nm UV light. ZEA was confirmed with benzidine diazonium salt [29].

Adsorptive stripping square-wave voltammetry (ASSWV) [13, 14, 23] combined with the standard addition method [25] was used for electrochemical quantification. Particularly, the standard addition method was chosen due to its advantages when interference from other substances is present [25]. Details of the protocol employed to perform ASSWV are given in Section 3.2.1.

For recovery experiments, uncontaminated maize extracts were spiked with the required amount of standard toxin and analysed by UV-vis absorption spectra as well as by the electroanalytical method proposed in this work. Each experiment was done at least in duplicate.

2.5. Apparatus and Measurements

UV-vis spectra were registered immediately after the preparation of solutions by using a Hewlett-Packard model 8452A spectrophotometer equipped with a temperature controller.

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The measuring system for the electrochemical techniques was composed of an EG&G PARC Model 273 potentiostat/galvanostat equipped with PAR270 electrochemical analysis software. Parameters used to obtain square-wave voltammograms were as follows: square-wave amplitude, $\Delta E_{SW} = 25$ mV, staircase step high, $\Delta E_S = 5$ mV and frequency, $f = 100$ Hz. In all these experiments the mycotoxin accumulation step for stripping analysis was accomplished in stirred solutions. Optimum parameter values are given below.

Electrochemical measurements were performed in a two compartment Pyrex cell [30]. The working electrode was a GC disk. Experiments were performed at 25 ± 1 °C. Other experimental details are described elsewhere [22].

3. Results and Discussion

3.1. Spectrophotometric Studies

The absorption spectra of commercial ZEA at different concentrations were recorded in ACN. Plots of both A^{274} and A^{316} vs c^*_{ZEA} were linear in a range from 0.955 ppm to 10.2 ppm. Extinction coefficients (ε) at the given wavelengths were $\varepsilon^{274} = 13111$ M^{-1} cm^{-1} and $\varepsilon^{316} = 6020$ M^{-1} cm^{-1}, respectively. Very small differences in the absorption spectra obtained in ACN with respect to those obtained in methanol ($\varepsilon^{274} = 13909$ M^{-1} cm^{-1} and $\varepsilon^{316} = 6020$ M^{-1} cm^{-1} [28] were observed. In addition, no significant differences were observed among spectra of either commercial ZEA or ZEA separated by TLC (ZEA_{TLC}) or even ZEA from extract solution (ZEA_{matrix}), showing that the presence of possible interferers do not disturb in a significant manner the spectra of ZEA. Therefore, concentrations of both ZEA_{matrix} and ZEA_{TLC} were calculated from these working curves.

3.2. Electrochemical Studies

3.2.1. ZEA from Commercial and Rice Samples

It is well-known that rice is a culture that is rarely contaminated by ZEA. Anyway, it is one of the grains usually chosen as a good substrate to obtain ZEA from inoculation with *Fusarium*, with a good performance [26]. It is also known that the behaviour of ZEA obtained by this way is similar to the behavior observed for the commercial one, at least from spectrophotometric measurements [26]. Thus, initial experiments were performed with commercial ZEA and ZEA produced in rice as both, either ZEA_{matrix} or ZEA_{TLC}.

ASSWV coupled with the standard addition method was used to perform the quantitative determination of ZEA preconcentrated on the GC electrode surface from solutions prepared from the commercial reagent and also from solutions prepared from ZEA obtained from inoculated rice samples.

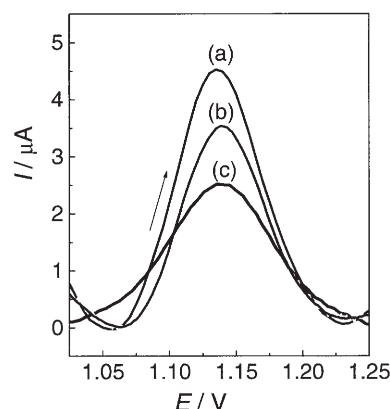

Fig. 2. Typical square-wave voltammograms for quantitative determination of ZEA preconcentrated on the GC electrode surface. a) Commercial reagent, $C^*_{ZEA} = 3.82$ ppm; b) ZEA_{TLC}, $C^*_{ZEA} = 3.25$ ppm; c) ZEA_{matrix}, $C^*_{ZEA} = 7.39$ ppm.

The accumulation time (t_{acc}) most favorable was 120 s and the accumulation potential (E_{acc}) for commercial ZEA and ZEA_{TLC} was any value in the range from 0.00 to 0.90 V (vs ECS). In ZEA_{matrix} determination reproducible results were difficult to be obtained for low E_{acc}, probably due to matrix interference. The best E_{acc} for performing the selective quantitative preconcentration of ZEA_{matrix} at the electrode surface was 0.90 V (vs ECS). Typical square-wave voltammograms are shown in Figure 2 (a) – (c).

Two solutions were prepared from extract solution by dilution: solution A ($c^*_{ZEA} = 80.9$ ppm) and solution B ($c^*_{ZEA} = 46.8$ ppm). The ZEA concentrations were determined by UV-vis spectrophotometric measurements as described in Section 3.1. ZEA of solution A was obtained after separation from the extract by TLC and then dissolved in ACN. A solution of $c^*_{ZEA} = 121$ ppb (solution A1) was then obtained by dilution of A. Solution B1 (117 ppb) was obtained by dilution of solution B. Both solutions were then analyzed for the quantification of both ZEA_{TLC} and ZEA_{matrix}, respectively, by employing the electroanalytical method proposed in this work. At least six additions of standard ZEA were spiked to the reaction medium. Square-wave voltammograms were recorded after the preconcentration step at the electrode surface performed at the t_{acc} and E_{acc} previously indicated for both ZEA_{TLC} and ZEA_{matrix}.

Concentration values calculated for ZEA_{TLC} and ZEA_{matrix} from linear regression parameters were (130 ± 22) ppb and (119 ± 6) ppb, respectively. Linear regressions can be expressed by a least square procedure as:

$$ZEA_{TLC} \; I_p = (0.24 \pm 0.04) + (5.89 \pm 0.07) \times 10^5 \; c^*_{ZEA} \quad (r = 0.999)$$

$$ZEA_{matrix} \; I_p = (0.41 \pm 0.01) + (1.10 \pm 0.03) \times 10^6 \; c^*_{ZEA} \quad (r = 0.995)$$

where I_p is expressed in amperes and c^*_{ZEA} in mol dm^{-3} and the errors are standard deviations. Data used in the regression analysis are average of at least two replicated measurements. Differences calculated for both methods,

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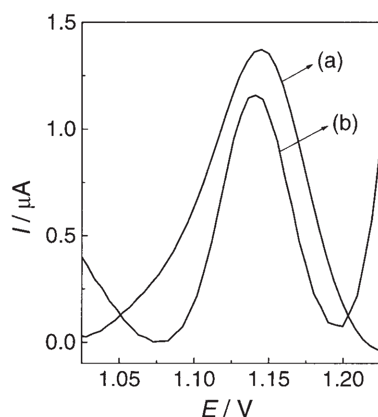

Fig. 3. Square-wave voltammograms of ZEA_{matrix} (a), C^*_{ZEA} = 637 ppb as well as ZEA_{TLC} (b), C^*_{ZEA} = 318 ppb, both obtained from maize inoculation, recorded in 20% ACN + 80% $HClO_4$ (aqueous solution) after preconcentration at E_{acc} = 0.90 V and t_{acc} = 120 s on a GC electrode.

i.e., spectrophotometric and electrochemical, are about 6% for ZEA_{TLC} and 2% for ZEA_{matrix}.

Results obtained by the electroanalytical technique are in good agreement with those calculated by spectrophotometric measurements of the same starting extracts and demonstrate the applicability of the proposed method to real fungal contaminated samples containing ZEA at sub-ppm level. These results demonstrate that ZEA could be also detected on rice without previous separating methods.

Table 1. Recovery of ZEA from spiked maize samples with no separation procedure by ASSWV/standard addition method on extracting solution.

ZEA added (μg kg^{-1})	Sample [a]	ZEA determined (μg kg^{-1})	Recovery (%)
52	1	56 ± 9	107
52	2	54 ± 8	103
104	3	105 ± 19	101
104	4	109 ± 19	105

[a] Each sample was spiked separately and assayed at least by duplicate.

3.2.2. ZEA from Maize Samples

Square-wave voltammograms of ZEA_{TLC} as well as ZEA_{matrix}, both obtained from maize inoculation, recorded in 20% ACN + 80% $HClO_4$ (aqueous solution) after preconcentration at E_{acc} = 0.90 V and t_{acc} = 120 s on a GC electrode are shown in Figure 3.

A well-defined ZEA_{matrix} peak was obtained in the complex sample without any previous separation. Good recovery percentage values from four samples of uncontaminated maize extracts spiked with ZEA were obtained, as shown in Table 1.

Recovery values slightly higher than 100% are obtained, in a remarkable agreement with those obtained by competitive direct monoclonal ELISA [31], ELISA with scFvQY1.5 (being scFvQY1.5 a specific recombinant antibody for ZEA) [6] and pressurized liquid extraction/LC-MS [8] on recovery of ZEA also from spiked corn. Reasons for these findings are not clear yet.

On the other hand, ZEA_{TLC} (fraction A) and ZEA_{matrix} (fraction B) were determined from extracts obtained from a given inoculated maize sample. The electrochemical analysis was performed for E_{acc} = 0.90 V (fraction A′ and B) and also for E_{acc} = 0.00 V (fraction A″). Results for A′ and A″ fractions were similar for both E_{acc}. The regression parameters of ASSWV coupled with standard addition method are given in Table 2.

ZEA concentration obtained by both the electroanalytical method proposed here and the spectrophotometric measurements are given in columns 4 and 5 of Table 2, respectively. They agree quite well for both, ZEA_{TLC} (fraction A′ and A″) and ZEA_{matrix} (fraction B). It is worthy to indicate that values of c^*_{ZEA} of column 5 are calculated from the value of c^*_{ZEA} of stock solution, which is in the range 40 – 50 ppm. Detection limit of absorption method is quite higher (≈950 ppb) than that found by ASSWV coupled with standard addition method (see below).

When starting from a given inoculated maize sample, we have found that about 60% of ZEA was recovered from the fraction separated by TLC as compared with the equivalent one made from de extract itself. These experiments allowed us to know that an important amount of mycotoxin is lost during the separation procedure.

Table 2. Determination of ZEA concentration from a given inoculated maize sample by ASSWV/standard addition method on preconcentrated ZEA on a glassy carbon electrode. A′: fraction separated by TLC and preconcentrated at E_{acc} = 0.90 V; A″: fraction separated by TLC and preconcentrated at E_{acc} = 0.00 V; B: fraction with no previous separation procedure preconcentrated at E_{acc} = 0.90 V.

Fraction	10^{-5} m (s) [a] (μA dm^3 mol^{-1})	B (s) [b] (μA)	c^*_{ZEA} (s) [c] (ppb)	c^*_{ZEA} [d] (ppb)	n [e]	r [f]
A′	4.3 (0.1)	0.40 (0.06)	296 (51)	301	5	0.997
A″	4.4 (0.1)	0.40 (0.06)	290 (51)	300	5	0.995
B	2.45 (0.43)	0.41 (0.04)	531 (146)	541	5	0.998

[a] m = slope of the linear regression of I_p vs c^*_{ZEA} plot. s: standard deviation.
[b] B = intercept of the linear regression of I_p vs c^*_{ZEA} plot. s: standard deviation.
[c] c^*_{ZEA} = concentration of ZEA calculated from the ASSWV/standard addition method. s: standard deviation (average standard deviation: ca. 20%).
[d] c^*_{ZEA} = concentration of ZEA calculated from absorbance measurements of concentrated extracts from which the different dilutions were prepared.
[e] n = number of additions of working solutions of ZEA for each sample. Every determination was performed at least by duplicate.
[f] r = correlation coefficient.

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Table 3. Determination of ZEA concentration from given maize samples by ASSWV/standard addition method on ZEA preconcentrated at $E_{acc} = 0.90$ V on a glassy carbon electrode. M1 (a)–(c) and M2 (a),(b) samples were prepared by dilution of working solutions prepared from extracts obtained from different inoculated maize samples. Determinations of ZEA were performed on M's samples with no previous separation procedure.

Sample	10^{-5} m (s) [a] (μA dm^3 mol^{-1})	B (s) [b] (μA)	c^*_{ZEA} (s) [c] (ppb)	c^*_{ZEA} [d] (ppb)	n [e]	r [f]
M1a	5.2 (0.2)	0.40 (0.06)	245 (44)	276	5	0.993
M2a	6.0 (0.3)	0.4 (0.1)	213 (64)	248	6	0.997
M1b	10.1 (0.4)	0.65 (0.04)	204 (21)	248	5	0.995
M2b	14.5 (0.5)	0.6 (0.1)	130 (26)	108	5	0.995
M1c	19.8 (0.8)	0.07 (0.01)	11 (1.9)	10	6	0.993

[a] m = slope of the linear regression of I_p vs c^*_{ZEA} plot. s: standard deviation.
[b] B = intercept of the linear regression of I_p vs c^*_{ZEA} plot. s: standard deviation.
[c] c^*_{ZEA} = concentration of ZEA calculated from the ASSWV/standard addition method. s: standard deviation (average standard deviation: ca.19%).
[d] c^*_{ZEA} = concentration of ZEA calculated from absorbance measurements of concentrated extracts from which the different dilutions were prepared.
[e] n = number of additions of working solutions of ZEA for each sample. Every determination was performed at least by duplicate.
[f] r = correlation coefficient.

ASSWV coupled with standard addition method was also tested for the determination of ZEA contents in two different inoculated maize samples. The ZEA concentrations of each corresponding concentrated extract were 1353 ppm and 1391 ppm, respectively, as determined by UV-vis spectrophotometry. From these extracts, stock solutions (M1 and M2) of 54.1 ppm and 43.6 ppm, respectively, were then prepared by dilution. Working solutions M1 (a)–(c) and M2 (a)–(b) were also prepared from M1 and M2 by dilution. ASSWV coupled with standard addition method was then performed on these working solutions. Results and linear regression parameters of the linear plots obtained are given in Table 3. The I_p vs c^*_{ZEA} plots were linear over the concentration range from about 20 to 3184 ppb. Good agreement was found between the c^*_{ZEA} values obtained from the method proposed here (Table 3, column 4) and those from UV-vis measurements (Table 3, column 5). Standard deviations in intercepts (column 3 of Tables 2 and 3) are practically independent of the solution used, i.e., either ZEA_{TLC} or ZEA_{matrix} from different samples, while standard deviations in slopes significantly differ for solutions of ZEA_{TLC} ($\approx$2.3% average, Table 2, column 2, fractions A′ and A″) and those of ZEA_{matrix} ($\approx$6% average, Table 2, column 2, fraction B and Table 3, column 2). This difference probably reflects the effect of the matrix itself.

Although the average of the percentual relative standard deviation of the ZEA concentrations is relatively high (about 20 %, see column 4 of Table 2 and 3), the percentual relative error for the method is rather low, i.e., $\approx -2.5\%$, as calculated by comparing values of c^*_{ZEA} of column 4 with those of column 5 of Tables 2 and 3.

The minimal concentration that could be estimated by the electroanalytical method proposed here was about 16 ppb for ZEA_{matrix} and about 13 ppb for ZEA_{TLC}. The experimental detection limit (dl) determined as the minimal concentration of ZEA_{Matrix} for a signal to noise ratio of 3:1 was dl = 30 ppb, which is about half of that obtained previously for ZAN by us [19]. These values could be compared favourably with some reported in the literature from chromatographic techniques such as Minicolumn, TLC and HPTLC (35–300 ppb) [5, 32–35] and also from the official method (HPLC, fluorescent detection, > 50 ppb) [36]. When lower detection limit techniques are required, HPLC or GC-MS with different cleanup and sometimes derivatization procedures [5, 8] or immunoassay methods [6] have to be chosen ($\approx$1–4 ppb), but they usually includes inherent disadvantages such as a strong commitment of time, labor and expense [6].

4. Conclusions

The application of square-wave voltammetry combined with adsorptive accumulation and the standard addition method is studied in this work to detect and quantify zearalenone (ZEA) mycotoxin that frequently contaminates maize and foodstuff. It has been proved that the methodology proposed is capable of quantifying ZEA at very low levels ($\approx$30 ppb) directly in extracting solution from contaminated rice as well as maize samples without previous separating procedures. Thus, it appears as a very promising analytical tool for the determination of ZEA in real samples. Moreover, it is expected that this method could be suitable for the analysis of other mycotoxins in contaminated real samples by considering that some of them, i.e., ZAN, CER, ATX-I are electroactive and show surface-active properties as well.

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