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Validation using QuEChERS method, risk assessment and preharvest intermission using GC-MS for determination of azoxystrobin in tomato and cucumber

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Abstract

Azoxystrobin residues in tomatoes and cucumbers was accurately determined by gas chromatography / mass spectrometry (GC-MS) in the selective ion monitoring (SIM) mode (the selected ion: m/z 344, 372, 388 and 403). The average recoveries at three levels came to range from 83.92% to 95.77% with a relative standard deviation of less than 20.0%. The limit of detection and the limit of quantitation were 0.01 and 0.05 mg kg^{-1}. Furthermore, the residues in tomatoes and cucumbers were estimated over 15 days. As for half-lives, they were shown to be rated between 1.69 and 4.5 days for both tomatoes and cucumbers after the application with recommended and double recommended dose, and found to be less than maximum residue limit so it can be recommended as preharvest gap. The dietary intake was not found to be abiding by the maximum permissible intake for both recommended or even double recommended dose, as it was found that both were less than the maximum permissible intake; which leads a vast improvement in azoxystrobin human health safety satisfaction.

Keywords: Risk assessment, PHI, Method validation,QuEChERS, GC-MS, Azoxystrobin

1. Introduction

Azoxystrobin (Fig.1) is a systemic, broad-spectrum fungicide belonging to the class of methoxyacrylates, which are derived from the naturally-occurring strobilurins. It exerts its fungicidal activity by inhibiting mitochondrial respiration in fungi. It is absorbed through the roots and translocated in the xylem to the stems and leaves, or through leaf surfaces to the leaf tips and growing edges. Azoxystrobin controls foliar and soil-borne diseases, including downy and powdery mildew, early and late blight, and the pathogens Sclerotinia, Alternaria, Ascochyta, Pythium, and Rhizoctonia on many crops [1].Due to the pesticide toxicity character, several countries have established maximum residue limits (MRL) for the presence of pesticide residues in crop products. The MRL is established independently in each country as pesticide registrations and is determinate through the result of toxicological and agronomic studies. These values may vary depending on the existing environmental conditions in the country, differing pest pressures, differing pesticide use patterns and good agricultural practices[2].In 2003, Anastassiades et al. introduced a sample preparation method named QuEChERS (quick, easy, cheap, effective, rugged and safe) involving pesticide dispersive solid phase extraction (dSPE), with primary secondary amine **(**PSA) sorbent [3]. Compared to other procedures, the QuEChERS method is very fast and cheap. This procedure has been used worldwide for studies on pesticide residue analysis in several matrices [4,5,6]. Several chromatographic techniques have been applied for the analysis of azoxystrobin in various matrices. These studies include gas chromatography [7,8,9,10], high performance liquid chromatography [11,12,13]. Moreover, gas chromatography–mass spectrometry (GC–MS) [14,15,16] and liquid chromatography-tandem mass spectrometry [17,18] have also been introduced in the analysis of azoxystrobin residues. Field application is usually done with the recommended dose in this work double recommended dose added to work to investigate the degradation and accumulation probability in the environment, and their relation to human health risk.

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Therefore, the objective of this study was to use the QuEChERS validated extraction method for the quantitative determination of azoxystrobin residues and its accumulation in tomato and cucumber samples applicated by recommended and double recommended dose conducting pre harvest interval(PHI), considering the possible matrix effects. Evaluating health risk assessment by conducting dietary exposure and maximum permissible intake.

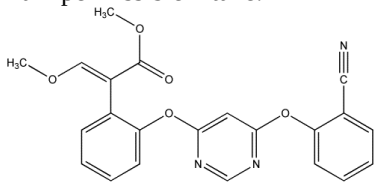

Figure 1. The chemical structure of azoxystrobin.

2. Material and methods

2.1. Chemicals and reagents

Acetone and acetonitrile were supplied by SDS (France) HPLC grade quality. Anhydrous magnesium sulfate and sodium chloride from El-Nasr pharmaceutical chemicals Co. (Egypt). And were activated by heating at 135 ºC overnight in the oven, cold and kept in desiccators until usage. QuEChERS salts 4 g MgSO4, 1 g NaCl, 1 g trisodium citrate dihydrate, 0.5 g disodium hydrogencitrate sesquihydrate, and d-SPE salts .Azoxystrobin reference standard Dr Ehrenstorfer GmbH (Augsburg, Germany) was supplied by Central Agricultural Pesticides Laboratory, Giza, Egypt. Formulation used in application is Blanc 25% SC, Sharda Worldwide Exports pvt. Ltd. India.

Individual solution (100 µg/mL) reference standard of azoxystrobin was prepared in acetonitrile in 100 mL volumetric flask. Analytical standard solutions were prepared for fortification and calibration purposes. Primary stock solutions were prepared from the pure reference materials (purity: 99.7%). Working solutions were diluted from the stock solutions or other working solutions. The additional standards were prepared and used as external calibration solutions for quantification. The successive working dilutions and spiking standard solution for GC-MS analysis were prepared daily. All standard and working solutions were stored at -18 ºC.

2.2. Instruments and apparatus

The determination of azoxystrobin residues in fruits and vegetables by gas chromatography/mass spectrometry (GC/MS) with the selective ion monitoring (SIM) mode (ions: m/z 344, 372, 388 and 403) [14,19]. The GC–MS analysis of azoxystrobin was performed with gas chromatography (HP6890 Series GC system) coupled to 5973 mass selective detector (Agilent Technologies, Inc., CA, USA) with detection system in the selective ion-monitoring mode (SIM) and the selected ions were 344, 372, 388 and 403 m/z. Sample ionization was achieved by electron impact at 70 k eV. The column used was an HP-5, 5% phenyl methyl siloxane (30 m * 0.25 mm * 0.25 µm). The oven was programed to start at 80 ºC for 3 min, ramp at 8 ºC/min until 280 ºC. The splitless injection of a 1 µl volume was carried out, with Helium as carrier gas with a flow rate (1 mL/min). The transfer line was held at 280 ºC. The retention time of azoxystrobin was 30.10 min. Helping apparatus were food shopper, Model 84181D, Hobart Corporation, Ohio, USA and high-speed cooling centrifuge, C-28 A 230-240V, 50-6- Hz, BOECO, Germany.

2.3. Field trials and sample preparation

The field trials, including the dissipation study were carried out at El-Daqahlya Governorate, Egypt. The plants at fruiting stage were sprayed with blanc 25% SC with 50 cm^3 /100 L water (recommended rate) and 100 cm^3 /100 L water (double recommended dose) for tomatoes and cucumber from the commercial products by knapsack hand sprayer (20L.) fitted with one nozzle boom. Control plots without pesticide application for each crop far away from treated plots at least 40m. . Replicate samples, 2 kg tomatoe and cucumber fruits were collected randomly from treated plots at intervals of one hour after application (zero time), 1, 3, 7, 10 and 15 days. As soon as the fruits were picked up, and put in polyethylene bags and transferred in ice box to the laboratory. Samples frozen first and then homogenized for at least 30 sec. The homogeneous matrix was stored at -20 ºC until the preparation day.

The samples were homogenized at low temperature (frozen) to avoid any significant influence of ambient temperature would result in degradation of pesticide . Frozen condition also helps to compensate for the heat generated when magnesium sulfate and sodium chloride were added. On the other hand, freezing-out removes most of the lipids, waxes and sugars as well as other components with low solubility in acetonitrile that may negatively affect the robustness of GC and LC analysis [3].

2.4. Extraction and clean-up

Extraction and cleanup were carried out according to the official method presented by Anastassiades and Lehotay 2003[4] . Ten g (±0.1 g) of the homogenated frozen sample were weighed in 50-mL centrifuge tubes. The extraction involved the addition of 10 mL of acetonitrile. The tubes were closed and vigorously

shaken by hand for 1 min. To induce separation and partitioning, salt mixture of 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride was added. The tubes were re-closed, vigorously shaken by hand for 1 min and vortexed, then centrifuged for 5 min at 3500g . The cleanup was carried out by transfering 1 mL of the acetonitrile phase into 15 mL centrifuge tubes containing 25 mg PSA and 150 mg anhydrous magnesium sulfate, the tube was vortexed for 1 min and then centrifuged for 5 min at 5000rpm. The supernatants were filtered using 0.2 µm PTFE filter (Millipore, Billerica. MA) into auto-sampler vials for GC-MS analysis.

2.5. Preparation of matrix-matched calibration solutions

Matrix effect comparing the response produced from the azoxystrobin in pure solvent solution with the samples were first extracted and then spiked with azoxystrobin in the same solvent at the same concentration level. The matrix effect was investigated by comparing the slopes of calibration curves at(0.01,0.1,0.2,0.5, 1.5,3 mg kg^{-1}) of azoxystrobin in tomatoes, cucumber matrices and in pure solvent.According to document [20]the acceptable drift between two bracketing injections of the same calibration standard should not exceed 30%.

2.6. Method Validation

According to Document [20], a within-laboratory method validation was performed to provide evidence that the method is fit for the extraction and quantitative determination of azoxystrobin in tomatoes and cucumber. Method validation is a requirement for accreditation bodies, and must be supported and extended by method performance verification during routine analysis where all steps that are undertaken in a method should be validated. The method was validated following a conventional validation procedure that included the following parameters: linearity, matrix effects, limits of quantification (LOQ), specificity, trueness (bias) and repeatability precision (RSDr).

2.6.1. Linearity

Multi-levels calibration (0.01, 0.1, 0.2, 0.5, 1.5, 3 µg/mL and calibration function were used. The fit of the calibrations was plotted and inspected by calculation of the residuals, avoiding over-reliance on correlation coefficient, to insure that the fit is satisfactory within the concentration range of the pesticides detected.

2.6.2. Matrix effect

Matrix effects were defined as the influence of one or more coextracted components from the sample on the measurement of azoxystrobin concentration. The presence of these effects is demonstrated by comparing the response produced from azoxystrobin in a simple solvent solution with that obtained from the same quantity of azoxystrobin in the presence of the sample or sample extract. Extracts of blank matrix (tomatoes and cucumber) used for preparation of matrix-matched calibration solutions at levels 0.01, 0.1, 0.2, 0.5, 1.5, 3 mg kg^{-1} were used to compensate the matrix effects for GC–MS analysis. Matrix effects (%ME) were calculated using the equation:

$$\text{ME }\% = \frac{\text{M matrix} - \text{M solvent}}{\text{M solvent}} X100\%$$

Where ME is the matrix effect, and M matrix and M solvent are the slopes of calibration curves in the matrix and in the pure solvent, respectively.

2.6.3. Limit of quantification LOQ

The limit of quantitation (quantification) was defined as the lowest concentration of the azoxystrobin that has been validated with acceptable trueness (70–120%) and precision (RSDr ≤ 20%) by applying the complete analytical method. According to the Sanco [20], the Limit of quantification should be ≤MRL. The maximum residue limit (MRL) for azoxystrobin is 3 and 1 mg kg^{-1} for tomatoes and cucumber, respectively [21.22] Specificity was defined as the ability of the detector (supported by the selectivity of the extraction and clean-up) to provide signals that effectively identify the analyte (azoxystrobin), these signals should be at levels ≤ 30% of RL (reporting limit). Absolute numbers and at this level the detector provide signals that effectively identify azoxystrobin. It is equal to or higher than the LOQ.

2.6.4. Trueness (bias)

The measure of trueness is normally expresses as ‘‘bias’’. It was defined as the closeness of agreements between the average values obtained from a series of test results (the mean recovery). Five replicates were used to check the recovery at the levels (0.1, 1, 3 mg kg^{-1}). According to the document [20] acceptable mean recoveries are those within the range of 70–120%. Trueness was calculated using the following equation:

$\%R = (X/\mu) \times 100$

%R: recovery percentage:

X: experimental concentration of azoxystrobin (mg kg^{-1}).

µ: calculated concentration of azoxystrobin (mg kg^{-1}):

2.6.5. Precision (RSDr)

The precision (Repeatability (r)) is defined as the standard deviation of measurement of azoxystrobin obtained using the same method on the same samples in a single laboratory over a short period of time, during which differences in the materials and equipment used and analysts involved will not occur. The value of ≤20% was used as the limit for RSDr. Five replicates for each recovery levels (0.1, 1, 3 mg kg^{-1}) per day on three different days were used to check the precision.
% RSD = deviation of the replicates /mean value of the replicates) x 100.

2.6.6 Risk assessement

In order to ensure food safety, risk assessement was conducted by calculating dietary exposure by multiplying each sample residue (mg kg^{-1}) for both tomato and cucumber by the average daily consumption for both too.Then rationalized by maximum permissible intake (MPI)which is found by multiplying ADI by mean body weight of an adult. [23,24,25].

3. Results and discussion

3.1. Method validation

3.2.1. Linearity

The evaluation of calibration curve linearity of azoxystrobin was done based on injections of standard solutions prepared in organic solvent (ethyl acetate) at concentrations 0.01, 0.1, 0.2, 0.5, 1.5, 3 mg kg^{-1} for GC–MS analysis. Figure 2 shows that the fit of the calibration is satisfactory.

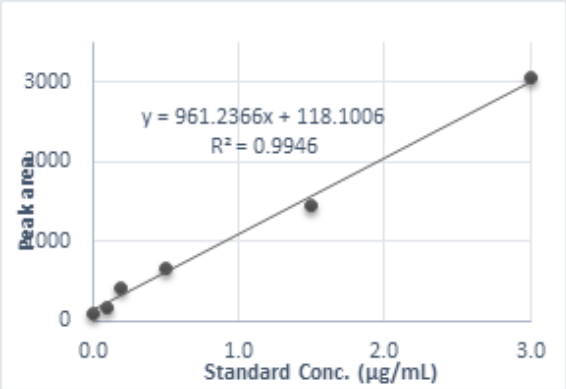

Fig.2. Calibration curve of azoxystrobin with GC-MS analysis.

3.1.2. Matrix effect (ME%), Limit of quantification LOQ

The matrix-matched calibration solutions were used to circumvent errors associated with matrix-induced enhancement and suppression effects in GC determinations. The matrix effect was evaluated by comparing the slopes of calibration curves (at levels 0.01, 0.1, 0.2, 0.5, 1.5, 3 mg kg^{-1}) of azoxystrobin in matrix (tomatoes and cucumber) (fig. 3) and in a pure solvent. The matrix effect for GC–MS analysis for both tomatoes and cucumber were 6.5 and 0.56 %, respectively.

The negative values of ME% for both tomatoes and cucumber for GC–MS analysis reflect matrix induced suppression. According to SANCO[20] **,** the acceptable drift between two bracketing injections of the same calibration standard should not exceed 30%. The lowest validated level of azoxystrobin with acceptable precision and trueness (LOQ) was 0.05 mg kg^{-1} for GC–MS analysis in both tomatoes and cucumber. The LOQ values are acceptable where LOQ ≤ MRL (3 and 1 mg kg^{-1}).

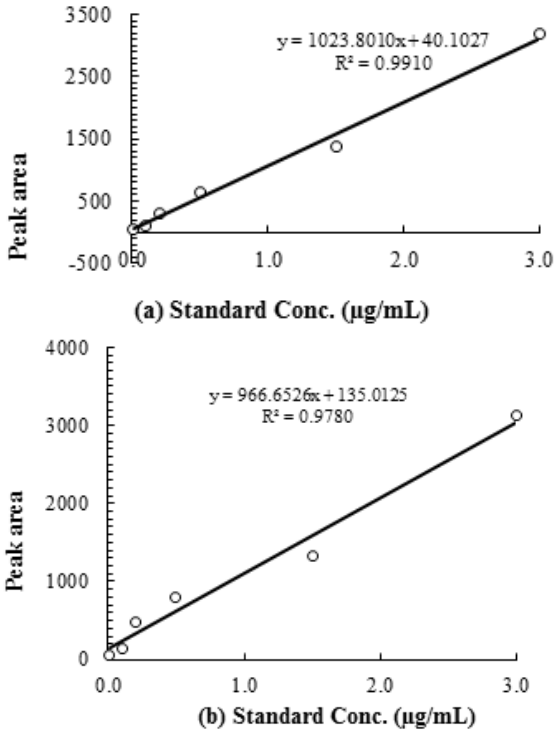

Fig.3. Matrix effect calibration curve of azoxystrobin in (a) tomatoes and (b) cucumber with GC-MS analysis

3.1.3. Trueness and precision (RSDr)

The trueness, bias or mean recovery was carried out in five replicates at levels (0.1, 1, 3 mg kg^{-1}) by spiking 10 g of blank sample with standard solutions. The

mean recoveries for tomatoes ranged from 83.92 to 92.34% with RSD ranging from 1.98 to 4.55. For cucumber, mean recoveries ranging from 86.17 to 95.77% with RSD ranging from 1.06 to 6.40. the obtained mean recoveries were within the acceptable range (70–120%).
The repeatability precision (RSDr) involved repeat of recovery levels (0.1, 1, 3 mg/kg), five replicates for each level per day on three different days. For GC–MS analysis, the (RSDr) values ranged from 6.22% to 16.38 % and from 5.01% to 12.84 % for tomatoes and for cucumber, respectively. The obtained (RSDr) values were within the acceptable range <20% .

3.2. Dissipation of azoxysrtobin in tomato and cucumber fruits

The results of dissipation of azoxystrobin on tomatoes and cucumber applicated at recommended dose and double recommended dose are presented in Table 1. The dissipation of pesticides residues in plants depends on the climatic conditions, type of application, plant species, dosage, the intervals between application and harvest [26] .No residues were found in the control samples collected from control plots of the experiment. Residue decline may be attributed to volatilization that occurred during the first days following application, removal by weathering, heat decomposition, sunlight and/or UV radiation [27,28]. Additionally, the growth dilution factor might have played a significant role [29].
Data of both crops were subjected to statistical analysis [30] for calculation of half -life ($t_{1/2}$) and waiting period (PHI). $t_{1/2}$ of azoxystrobin on tomatoes and cucumber fruits were found to be 3.61and 1.69 days, respectively at recommended dose. whereas, data revealed that at 4.5 and 2.2 days at double recommended dose for tomatoes and cucumber fruits respectively. Residues were below the MRL limit (codex alimentarius commission 2019) which it can be suggested for growers as harvest gap.

Our results were agreed with Sundravadana [31] who reported that the half life of azoxystrobin in mango, when sprayed at the recommended dose (1.0 mL *per* L) was one day and residue dissipates within three days after spray.
Also the results of Montasser and. Mahmoud [32] who found the pre-harvest intervals (PHI) were 6 days after application of azoxystrobin on grapes while the half life values 3.01 and 2.8 days for grape leaves and fruits, respectively. The initial deposits of azoxystrobin were 4.85 mg kg^{-1} and 1.86 mg kg^{-1} in leaves and fruits of treated grapes, the residues declined to 0.12 mg kg^{-1} on fruit after 10 days of application, and it was undetectable after 21 days. Only 0.54% of the initial deposit was detected on fruit after 15 days. The residues on grape leaves declined to 0.59 ppm after 10 days to represent 12.17% of the initial deposit.

Results of [33] showed that residues of azoxystrobin on Chinese cabbage declined from 4.10 to 0.63 mg kg^{-1} within 18 days, and from 13.21 to 0.10 mg kg^{-1} within 9 days on Chinese kale. The safe harvest intervals were suggested to be 15 and 10 d after the last application for Chinese cabbage and leafy vegetables, respectively.
Azoxystrobin residues were detected in cucumber fruits during the 8-day post application analysis [34] . The detected values varied depending on the amount applied and the time of residue detection. However, residues were below MRL after 4 days, which is consistent with the PHI mandated by regulation.

The differences in levels of initials deposits of pesticides on both vegetables, tomato and cucumber are mainly due to many factors; the ratio of surface to mass area and character of treated surface, smooth or rough and waxy or non-waxy[35]**.** The systemic character of tested compound, high wax content of tomato fruit surface and **hydrophilic**-lipophilic balance of investigated pesticide controlled the penetrability of applied agrochemicals into fruit tissues [36]. Degradation and dissipation residues of azoxystrobin from tomato and cucumber fruits happened because the initial deposits and residues at different intervals of this pesticide are influenced by different factors: evaporation of the surface residue which is dependent on temperature condition, biological dilution which is dependent on the increase mass of fruits, chemical or biochemical decomposition, metabolism and photolysis.
Great interest to note that the same factors were studied by several investigators. Christensen [37] reported that the decline of pesticides may due to biological, chemical or physical processes, or if still in the field, due to dilution by the growth of the crop.

Plant growth, particularly for fruits is also responsible to a great extent for decreasing the pesticide residue concentrations due to growth dilution effects [38] . In addition, the rapid dissipation of originally applied pesticide is dependent on a variety of environmental factors such as sunlight and temperature [39,40] . However, high temperature is reported to the major factor in reducing the pesticides from the plant surface

[41]. Light plays an important role in the behavior of pesticide in the environment [42].

Table 1. Azoxystrobin residues in both tomatoes and cucumber with recommended and double recommended dose.

Intervals after treatments(days)	Tomatoes				Cucumber			
	Residues (mg kg^{-1})				Residues (mg kg^{-1})			
	Recommended dose	Dissipation%	Double recommended dose	Dissipation%	Recommended dose	Dissipation%	Double recommended dose	Dissipation%
0	2.75±0.51	0.00	5.99±0.31	0.00	2.23±0.21	0.00	5.02±1.89	0.00
1	2.05±0.32	25.46	4.89±0.69	18.37	1.22±0.19	45.30	2.87±1.49	42.83
3	1.59±0.22	42.18	4.02±0.60	32.89	0.35±0.15	84.31	0.82±0.83	83.67
7	0.7±0.12	74.55	1.79±1.24	70.20	0.10±0.08	95.52	0.33±0.18	93.43
10	0.33±0.05	88.00	0.88±0.35	85.31	0.05±0.06	97.76	0.24±0.34	95.22
15	0.05±0.04	98.20	0.25±0.21	95.90	ND	ND	0.08±0.10	98.50
Codex MRL	3				1			

Table(2) Regression equation, Correlation coefficient and half-life for both recommended and double recommended dose for Azoxystrobin in tomato and cucumber.

Dosage	Tomato		Cucumber	
	Recommended dose	Double recommended dose	Recommended dose	Double recommended dose
Regression equation	y = -0.1105x + 0.5	y = -0.0911x + 0.8241	y = -0.1629x + 0.2212	y = -0.1131x + 0.4919
Correlation Coefficient (R^2)	0.9704	0.9888	0.9649	0.9336
Decomposition rate(K)	0.19	0.15	0.4	0.31
t 1/2 (days)	3.61	4.5	1.69	2.21

Table (3) Maximum permissible intake and dietary exposure for Azoxystrobin at recommended dose and double recommended dose.

Days after application	Maximum Permissible Intake (MPI)mg person^{-1} day^{-1}	Tomato		Cucumber	
		Residues mg kg^{-1}	Dietary exposure mg person^{-1} day^{-1}	Residues mg/kg	Dietary exposure mg person^{-1} day^{-1}
Recommended dose			0.227975	2.23	0.184867
0	12	2.75			
1	12	2.05	0.169945	1.22	0.101138
3	12	1.59	0.131811	0.35	0.029015
7	12	0.70	0.058030	0.10	0.008290
10	12	0.33	0.027357	0.05	0.004145
15	12	0.05	0.004145	N.D.	N.D.
Double recommended dose					
0	12	5.99	0.496571	5.02	0.416158
1	12	4.89	0.405381	2.87	0.237923
3	12	4.02	0.333258	0.82	0.067978
7	12	1.69	0.140101	0.33	0.027357
10	12	0.88	0.072952	0.24	0.019896
15	12	0.25	0.020725	0.08	0.006632

3.3 Risk assessment: Dietary exposure at zero time from application
n found to be 0.22 and 0.49, 0.18 and 0.41 mg kg^{-1} for both tomato and cucumber at recommended and double recommended doses respectievely which is less than maximum permissible intake (MPI) 12 mg $person^{-1}$ day^{-1} [43,44,45]. , leads that azoxystrobin impact on food can be expressed as safe on human health.

4.Conclusion

Dissipation rate and half-life after one application with recommended dose and double recommended dose for azoxystrobin on tomatoes and cucumber under field conditions were conducted using validated QuEChERS method for sample preparation for determination using GC/MS. Half-life ($t_{1/2}$) found to be from 1.69 to 4.5 days less than MRL which can be conducted as preharvest gap. Risk assessement found dietary intake at zero time for both applications are less than maximum permissible intake which is humanly safe.

5. Conflict of Interest

The authors declare no conflicts of interest.

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