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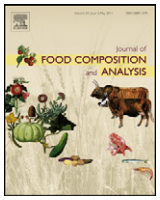

Original Article

Sigmoidal kinetics of anthocyanin accumulation during fruit ripening: A comparison between açai fruits (*Euterpe oleracea*) and other anthocyanin-rich fruits

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ABSTRACT

Anthocyanins are natural colorants with increasing interest. *Euterpe oleracea* fruits (EOF) (açai) are an interesting phenolic compounds source. They are extremely rich in two anthocyanins: cyanidin-3-glucoside and cyanidin-3-rutinoside. In this study, the anthocyanin content was evaluated in EOF during their ripening, allowing to characterize very important parameters for the post-harvest industry: their maximum accumulation rate, per day, (Δy), their maximum concentration in fruits (CMAX) and the corresponding maturity class (S). Samples of 12 racemes from three plantations were collected twice a month during their ripening process. The maturity class of EOF was recorded at each harvesting and their anthocyanin content was determined by an HPLC method after solvent extraction. Anthocyanin accumulation was described ($p < 0.05$) by sigmoidal equations and Δy, CMAX and S values were determined. The Δy of EOF reached 35.63 mg kg^{-1} fruits day^{-1}, whereas CMAX reached 1443 mg kg^{-1} fruits. On average, in the beginning of maturation, both anthocyanins were present in similar proportions. However, in the last maturity stages, cyanidin-3-glucoside became less abundant than cyanidin-3-rutinoside. On the basis of the data available on strawberries, grapes, pomegranates and lychees, it was possible to verify that the sigmoidal mathematical model of anthocyanin accumulation is transposable.

1. Introduction

Anthocyanins are the largest and most diverse group of plant pigments derived from the phenylpropanoid pathway. These pigments are responsible for many orange, pink, red, violet, and blue colors. They are part of a large and widespread group of plant flavonoids and water-soluble phenolic compounds. Anthocyanins are normally found dissolved uniformly in the vacuolar solution of epidermal cells. There are 20 anthocyanidins, differing in the number and position of their hydroxyl and methoxyl groups. Anthocyanidins are modified by glycosyl and aromatic or aliphatic acyl moieties, resulting in hundreds of anthocyanin molecules (Andersen and Jordheim, 2006; Castañeda-Ovando et al., 2009; Oren-Shamir, 2009). Several studies have revealed that anthocyanins and anthocyanin extracts are responsible for the high antioxidant activities of fruit and other food extracts (Orak, 2007; Du et al., 2008; Sun et al., 2009; Yang and Zhai, 2010).

In the kinetics of anthocyanin (Ribereau-Gayon, 1982), lycopene (Arias et al., 2000), soluble solids and sugars (Bonvehi et al., 1997) accumulation, three stages were observed: a phase of progressive increase in concentration, a transitional phase characterized by a decreasing accumulation rate leading to a maximum concentration, and a phase of decreasing concentration. The final decreasing phase should not be considered, as the observed phenomenon is due to oxidation or degradation and not synthesis. In most of the cases, the best mathematical model that describes such an accumulation profile is the sigmoidal model (Woodward, 1972; Rogiers and Knowles, 1997; Fernández-López et al., 1999; Rivera-López et al., 1999; Wang and Lin, 2000).

Euterpe oleracea is a palm tree widely distributed in northern South America, with its greatest occurrence and economic importance in the floodplains of the Amazonian delta. *E. oleracea* fruits (EOF) are round-shaped drupes (diameter of about 12 mm) associated in racemes. For the most popular variety, the fruit color

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goes from green to black during the ripening process. Fully ripen fruits are in addition covered with a wax cuticle (Rogez, 2000). The exocarp is a thin layer and the mesocarp has a thickness of only 1–2 mm; the kernel represents around 85% of the volume of the fruit (Pompeu et al., 2009b). The ripen fruits have a very high content in phenolic compounds and notably anthocyanins (mainly cyanidin-3-glucoside, C3G, and cyanidin-3-rutinoside, C3R) (values ranging from 0.50 to 10.20 mg g^{-1} of dried pulp) (Rogez, 2000; Gallori et al., 2004; Schauss et al., 2006b). They present substantially higher values of antioxidant activity than most any fruits (Lichtenthaler et al., 2005; Schauss et al., 2006a; Pompeu et al., 2009b).

The kinetics of anthocyanin accumulation in plants has been poorly documented. Some studies report on changes and increase in anthocyanin content with time or stage of maturity but do not determine the kinetics of the phenomenon (Mozetič et al., 2004; Kulkarni and Aradhya, 2005; Usenik et al., 2009). The characterization of anthocyanin accumulation kinetics is very relevant because it allows defining the maturity stage during which synthesis of the pigments is maximum, as well as the one for which the maximum concentration has been reached, both parameters being important for the post-harvest industry. Thus, the aim of the present work was to determine the kinetics of anthocyanin accumulation during EOF ripening and to verify if such kinetics can be transposed to other anthocyanin-rich fruits.

2. Materials and methods

2.1. Plant material

Fruits from 12 racemes of 6-year-old *E. oleracea* were harvested in the floodplains of the eastern Amazonian region (State of Pará, Brazil). Eight racemes were collected in the municipal district of Abaetetuba, four in each of two locations (A and B, 1°44′37.4″S, 48°55′01.2″W, and 1°47′36.10″S, 49°03′49.94″W, respectively), 17 km distant from each other. The other four racemes were collected on the Island of Cumbu, municipal district of Acará (Location C, 1°30′35.42″S, 48°27′55.70″W), 55.5 and 72 km distant from Locations A and B, respectively. Twice a month, two rachillae of each stem were randomly cut with a knife at 2 cm of the main rachis: the first one in the terminal zone and the second in the initial zone. The two rachillae of the same stem were mixed to make one unique sample. Rachillae were handled with sterile gloves and packed in sterile bags. Individual fruits of the same rachilla could be at different maturity stage (from completely green to black and fully ripe). The exact maturity stage (S) of each sample was determined on the basis of the percentage of the predominant color, counting the exact number of fruits of each color. The start of the ripening process was defined by the existence of the first black fruits on the raceme. The following scale was used to classify the samples in terms of maturity stage: stages 1–6, green fruits with, at least, 0%, 20%, 40%, 60%, 80% and 100% of black fruits, respectively; stages 7–11, black fruits with a minimum of 20%, 40%, 60%, 80% and 100% of fruit covered with wax cuticle, respectively. Samples were quickly transported to the laboratory and were immediately processed.

2.2. Chemicals

Methanol and formic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Cyanidin-3-glucoside and cyanidin-3-rutinoside were acquired from Extrasynthèse (Genay, France). All other chemical reagents were of HPLC grade and were purchased from Synth, Merck and CRQ (all from São Paulo, Brazil).

2.3. Extraction and quantification of anthocyanins

Samples of EOF (30 randomly-chosen drupes) were weighed (42.1–52.0 g) and introduced in a 150 mL brown glass bottle containing 100 mL acidified ethanol (1% of 12 M HCl, v/v). After 48 h of extraction at 25 °C without stirring, the solution was collected, filtered through a Whatman no. 1 filter (Whatman Inc., Clifton, NJ) and assayed for anthocyanins by an HPLC method.

2.4. HPLC analysis of anthocyanins

The anthocyanins were separated by HPLC, using a Lichrospher RP-100 (C_{18}, 100 Å pores, 5 μm particle size) column (125 mm × 4 mm) preceded by a guard column (10 mm × 4 mm). The HPLC system consisted of a P-1000 Thermo Separation Products pump (San Jose, CA), a thermostabilisation system (Spectra-Physics, Oxfordshire, UK) fixed at 30 °C, a UV/Vis LC 290 detector (Perkin Elmer, Wellesley, MA) and a DP800 Carlo Erba Instruments (Milan, Italy) integrator. Two solvents were used: 1% formic acid in ultra-pure water (solvent A) and acetonitrile (solvent B). Twenty μL samples were injected. Elution was achieved with a gradient, starting with (v/v) 20% B to reach 35% B at 20 min. The mobile phase was then returned to the initial condition in 5 min and maintained for 5 additional min. The rate of solvent flow was kept constant at 1 mL min^{-1} throughout the procedure. Chromatograms were recorded at 510 nm. For quantification, standards (C3G (retention time: 10.5 min) and C3R (retention time: 11.7 min)) were used and the calibration curves were plotted for each standard compound on the basis of peak area. The total anthocyanin content was expressed in mg kg^{-1} of fresh fruits. Triplicate tests were conducted on each collected sample of rachillae.

2.5. Data analysis

Data are presented as the mean values of the measurements performed with the four different stems in each one of the three independent locations. The changes in total anthocyanin content were fitted to sigmoidal curves (Nelder, 1961) with version 3.02 of the GraphPad PRISM Software (GraphPad Software Inc., San Diego, CA). The fit converged for all data sets. Sigmoidal curves started at 0.0, presented a plateau at C_{MAX} (maximal concentration in product) and a variable slope factor. They all corresponded to Eq. (1):

$$Y = \frac{C_{MAX}}{1 + 10^{(X_{1/2} - X) \times g}} \quad (1)$$

where Y is the concentration in product of the reaction, g is the gain term (Hill slope), $X_{1/2}$ is the time (in days) at which $Y = C_{MAX}/2$, X is the maturation time (in days) after the first black fruits appear. Software options were: each replicate was considered individually, the non-linear regression was made without weighting, minimize absolute distances squared, derivatives were calculated with a lower but, more accurate method, convergence was reached when two consecutive iterations changed the sum-of-squares by less than 0.01%.

3. Results and discussion

3.1. Anthocyanin accumulation

The EOF presented great differences in extent and kinetics of anthocyanin accumulation with regard to the harvesting places (Fig. 1). EOF from Locations A and C presented the shortest time of ripening and the highest final content in anthocyanins, while EOF from Location B showed lower and slower accumulation of anthocyanins. This wide range of variability in anthocyanin concentration between the three locations might be attributed

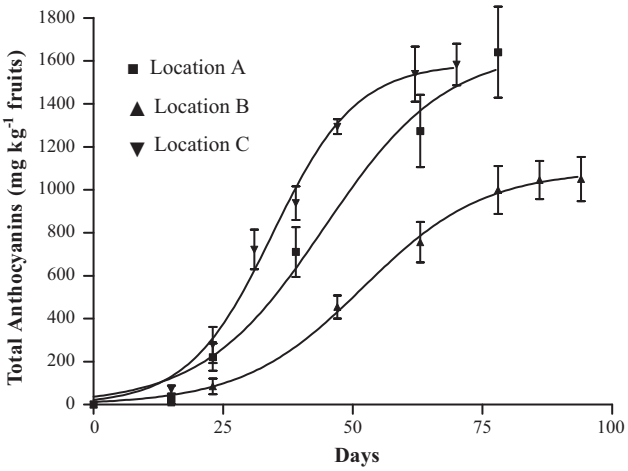

Fig. 1. Accumulation of total anthocyanins in *Euterpe oleracea* fruits (EOF) over a single season, at three different locations in the Amazonian floodplains (Locations A and B: Abaetetuba; Location C: Island of Cumbu). Field symbols represent the mean of four samples. Error bars indicate standard deviation. Full lines represent the best-fit sigmoidal lines.

to pedological factors, namely for Locations A and B, which are distant only 17 km, but neither to the climate, which was similar in terms of temperature, sunlight and rain falls, nor to the variety, which was the same.

The maximum anthocyanin concentrations (C_{MAX}) obtained in the present study ranged from 1090 to 1650 mg kg^{-1} of fruits (Table 1), with a mean value of 1443 mg kg^{-1} fruits, which means that these fruits can be considered as anthocyanin-rich (Pompeu et al., 2009b). Pompeu et al. (2009b) extracted anthocyanins from EOF under optimized conditions and obtained higher anthocyanin concentrations ranging from 2130 to 2681 mg kg^{-1} fruits. These levels of accumulation in the fruit pulp and peel appear quite high in comparison with other anthocyanin-rich fruits, especially if one considers that about 85% of the fruit volume is made of the kernel. Usenik et al. (2009) investigated the accumulation of five anthocyanins during ripening (25–33 days) of *Prunus domestica* ('Jojo', 'Valor', 'Čačanska rodna' and 'Čačanska najbolja' cultivars). Anthocyanin concentration ranged from 74.40 to 328.50 mg kg^{-1} of fresh plums at the end of the maturation. These fruits did not present a great increase in anthocyanin concentration during ripening and the differences were attributed to the cultivars. Mozetič et al. (2004) studied the anthocyanin content of the Slovenian sweet cherries (Petrovka cultivar) at 7 maturity stages. These fruits presented a high increase in total anthocyanin concentration (from 0 to 1150 mg kg^{-1} fruits in only 20 days after full bloom). Kulkarni and Aradhya (2005) observed a rapid increase in the anthocyanin concentration in pomegranate between 20 and 80 days of fruit development. The highest anthocyanin concentration (1380 mg kg^{-1} fruits) was recorded in 100-day-old fruits. Fernández-López et al. (1999) analyzed three red grape cultivars of different coloration during ripening (20–50 days after veraison). These fruits presented anthocyanin concentrations ranging from 52.5 to 1853 mg kg^{-1} fruits.

3.2. Accumulation kinetics of the anthocyanins in E. oleracea fruits

As shown in Fig. 1, the accumulation of total anthocyanins during the maturation of EOF could be fitted to sigmoidal curves. The R^2 values were above 0.95 for the three locations under investigation (Table 1). The time necessary to reach C_{MAX} is very long for EOF: between 69 and 94 days were necessary after the first black fruits appeared, depending on the location. For this reason, the Hill Slopes were very low for each location.

The time ($X_{1/2}$) necessary to reach half of the C_{MAX} corresponds to the inflexion point of the curves. At this point, it is possible to calculate the maximal accumulation rate of anthocyanins in the fruits: Δy expressed in mg of anthocyanin kg^{-1} fruits. EOF present Δy values varying between 23.24 and 48.60 mg^{-1} kg^{-1} fruits day^{-1} (mean = 35.63 mg^{-1} kg^{-1} fruits day^{-1}).

As anthocyanins are of great benefit to health and of interest for many applications in the post-harvest industry, it is not recommended to harvest the fruits near $X_{1/2}$ days. It would be very interesting for producers to know how many days they should wait before collecting the fruits after the first black fruits appear on the racemes. The time taken to reach 75%, 85% and 95% of C_{MAX} was calculated for each fruit in relation to $X_{1/2}$ ($a_{0.75}$, $a_{0.85}$ and $a_{0.95}$, respectively, Table 1). In the case of EOF, these times were respectively 1.27, 1.42 and 1.71 longer than the time necessary to reach half of C_{MAX}. We suggest that the EOF should be collected at 85% of C_{MAX}, which means 1.42 $X_{1/2}$ days after the appearance of the first black fruits. One should indeed consider on the one hand that the rate of accumulation decreases progressively when the anthocyanin concentration approaches C_{MAX} and on the other one

Table 1
Statistical data analysis of the sigmoidal curves (Fig. 1) of total anthocyanin content with ripening time (days) in *Euterpe oleracea* fruits (EOF) and other anthocyanin-rich fruits.

Fruits	C_{MAX}	$X_{1/2}$ (days)	Hill slope	Δy	R^2	n	$a_{0.75}$	$a_{0.85}$	$a_{0.95}$
EOF A[a]	1650 mg kg^{-1} fruits	44.5	0.0371	35.06	0.9596***	19	1.29	1.46	1.77
EOF B[a]	1090 mg kg^{-1} fruits	52.0	0.0372	23.24	0.9773***	28	1.25	1.39	1.66
EOF C[a]	1588 mg kg^{-1} fruits	34.5	0.0537	48.60	0.9813***	27	1.26	1.41	1.69
Mean of EOF	1443 mg kg^{-1} fruits	43.7	0.0427	35.63			1.27	1.42	1.71
Pomegranate-BA$_1$[b]	34 mg l^{-1} juice	25.2	0.2048	4.03	0.9971***	6	1.09	1.15	1.25
Black grape[c]	340 mg kg^{-1} fruits	9.6	0.1421	28.10	0.9782***	6	1.35	1.55	1.94
Lychee[d]	826 mg kg^{-1} pericarp	61.6	0.0295	14.01	0.9715***	9	1.26	1.41	1.70
Exotic grape[e]	701 mg kg^{-1} fruits	21.0	0.0425	17.16	0.9739***	8	1.53	1.84	2.43
Flame seedless grape[e]	50 mg kg^{-1} fruits	5.0	0.2317	6.66	0.9846***	6	1.41	1.65	2.10
Strawberry[f]	308 mg kg^{-1} fruits	14.7	0.1563	27.66	0.9986***	8	1.21	1.33	1.56

C_{MAX} is the maximum concentration of anthocyanin; $X_{1/2}$ is the time (in days) at which $Y = C_{MAX}/2$; Δy = daily variation of anthocyanin content (Y) at $X_{1/2}$; $a_{0.75}X_{1/2}$, $a_{0.85}X_{1/2}$, $a_{0.95}X_{1/2}$ are the times (in days) at which $Y = C_{MAX}$ 0.75, C_{MAX} 0.85, C_{MAX} 0.95, respectively; R^2 is the determination coefficient, n is the number of experimental values.

[a] First day of ripening was characterized by the first black fruits in a raceme.
[b] Based on experimental data from Hernández et al. (1999).
[c] Based on experimental data from Ribereau-Gayon (1982).
[d] Based on experimental data from Rivera-López et al. (1999).
[e] Based on experimental data from Fernández-López et al. (1999).
[f] Based on experimental data from Woodward (1972).
*** Significant value for $p < 0.001$.

Table 2
Statistical data analysis of the sigmoidal fitting (Fig. 2) of total anthocyanin changes in relation to the maturity class of *E. oleracea* fruits (EOF).

Fruits	C_{MAX} (mg kg^{-1} fruits)	$S_{1/2}$	Hill slope	R^2	n	$S_{0.75}$	$S_{0.85}$	$S_{0.95}$
EOF A[a]	1609	6.0	0.3201	0.9772***	21	7.49	8.35	9.99
EOF B[a]	1159	6.9	0.3048	0.9868***	27	8.47	9.37	11.10
EOF C[a]	1591	5.4	0.3255	0.9831***	26	6.87	7.71	9.33
Mean	1453	6.1	0.3168			7.61	8.48	10.14

C_{MAX} is the maximum concentration of anthocyanin; $S_{1/2}$ is the stage (from 1 up to 12) at which the anthocyanin content (Y) = $C_{MAX}/2$; $S_{0.75}$, $S_{0.85}$, $S_{0.95}$ are the stages for which $Y = C_{MAX}$ 0.75, C_{MAX} 0.85, C_{MAX} 0.95, respectively. R^2 is the determination coefficient, n is the number of experimental values.
[a] First day of ripening was characterized by the first black fruits in a raceme.
*** Significant value for $p < 0.001$.

that structural and microbiological disorders may appear with time (Pompeu et al., 2009a).

Accumulation kinetics appear to be highly dependent on the growing location, suggesting an effect of environmental factors, such as the type of soil, and the flood level, the intensity of the sun light or even the season. More studies are obviously needed to investigate these factors.

3.3. Comparison with other anthocyanin-rich fruits

On the basis of experimental data from the literature, we could perform a statistical data analysis related to the anthocyanin accumulation during ripening of other anthocyanin-rich fruits. Table 1 shows that, for each fruit, the accumulation of total anthocyanins could be fitted to a sigmoidal curve. In the case of lychee and Exotic grape, the Hill Slopes were in the same range as for EOF. They were, however, one order of magnitude higher for Flame seedless grape, black grape, strawberry and pomegranate-BA_1, indicating a much quicker phenomenon. Δy was similar (black grape, strawberry), slightly lower (lychee, Exotic grape) or much lower (Flame seedless grape, pomegranate-BA_1) than in EOF.

3.4. Relationship between anthocyanin content and maturity class

Producers usually evaluate the maturity of anthocyanin-rich fruits by looking at their external color, instead of counting the time after the start of fruit ripening (first black fruits). At each collection of EOF samples, we precisely determined the stage of maturity of the fruits on the basis of the proportion of black fruits or black fruits covered with a wax cuticle.

The anthocyanin concentrations were plotted against the stage of maturity. The experimental points could also be fitted to sigmoidal curves (Fig. 2). The corresponding equation parameters were determined and are presented in Table 2. The inflexion point corresponded to the stage of maximal anthocyanin accumulation (=$S_{1/2}$). Interestingly enough, a mean value of 6.1, mathematically corresponding to 100% black fruits, was obtained for $S_{1/2}$, meaning that only half of C_{MAX} was achieved. This clearly indicates that the external black appearance of the fruits does not correspond to their best maturity in terms of anthocyanin content. The maturity stage corresponding to an anthocyanin concentration reaching 75%, 85% and 95% ($S_{0.75}$, $S_{0.85}$ and $S_{0.95}$, respectively) of the maximal value could be evaluated (Table 2). An anthocyanin concentration of 0.85 C_{MAX} was obtained for a mean value of maturity class of 8.48. As the maturity class 9 is defined as a minimum of 60% of black fruits covered with a wax cuticle on their surface, producers should be encouraged to wait at least until that stage for the picking of EOF.

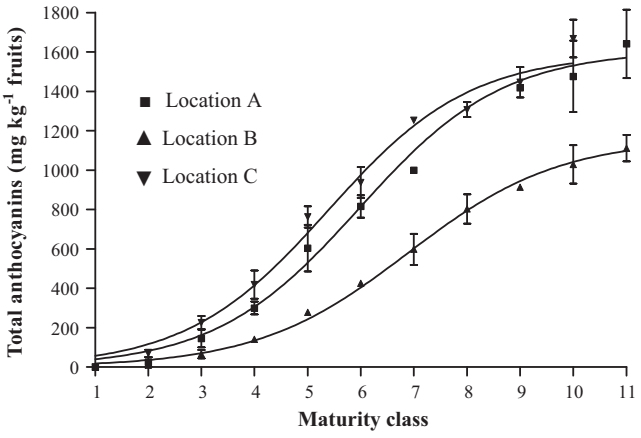

Fig. 2. Accumulation of total anthocyanins from *E. oleracea* fruits (EOF) according to the maturity stage, at three different locations in the Amazonian floodplains (Locations A and B: Abaetetuba; Location C: Island of Cumbu). Field symbols represent the mean of four samples. Error bars indicate standard deviation. Full lines denote the best-fit sigmoidal lines.

Traditionally, the Brazilian small farmers pick the fruits between maturity stages 5 and 11, depending on the time of year, which is a major factor influencing the price of EOF (Rogez, 2000). The food sector, which deals with the açai drink and/or their by-products such as clarified açai or extracts, should take both these economical aspects and the biological information included in the present study into account, in order to maximize the quality of their products for the consumer.

3.5. Anthocyanin profile

A change in anthocyanin profile in function of the maturity ripening was observed for all three racemes of EOF. The mean of anthocyanin concentration for three location at early (predominantly green, stages 1–3), intermediary (stages 6–8), and later maturity (stages 9–11) stages was: 90.70 mg (C3G over C3R ratio of 62.5 ± 8.5% and C3R: 37.5 ± 7.5%), 892.70 mg (44.7 ± 5.3%/55.3 ± 6.3%), and 1365.20 mg kg^{-1} fruits (23.1 ± 5.3%/76.9 ± 8.6%), respectively.

4. Conclusions

E. oleracea fruits are considered as rich in anthocyanins. These anthocyanins are made of cyanidin-3-glucoside and cyanidin-3-rutinoside, but the proportion of the latter increases with maturity. The accumulation of total anthocyanins during the maturation of anthocyanin-rich fruits could be fitted to sigmoidal curves in two ways: expressing concentration versus time (in days) or versus maturity stage. Mathematical expressions allowed us to determine the maximum concentration, the maximal accumulation rate of anthocyanins and the Hill slope. Time and maturity stage necessary to reach 75%, 85% and 95% of the maximum concentration of anthocyanins were calculated. As anthocyanins are of high benefit to health and of high industrial value, we recommend harvesting EOF when 85% of the maximum anthocyanin concentration is reached, which corresponds to the maturity stage 9, defined as having more than 60% of the fruits with a wax cuticle on their surface. The sigmoidal model developed to predict anthocyanin accumulation in EOF could be successfully used for data from

the literature on other anthocyanin-rich fruits. This paper underlines the interest for a clear knowledge of the kinetics of anthocyanin accumulation as it makes it possible to select the best stage for harvesting any anthocyanin-rich fruit, given that these polyphenols are of high interest.

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