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Determination of ascorbic acid in vegetables by capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) was used for the determination of ascorbic acid (AsA) in vegetables. AsA was extracted from vegetables by homogenizing with 2% thiourea–10 m*M* hydrochloric acid and determined directly by CZE with a borate buffer. The limit of detection (LOD) for AsA was 0.35 mg/l ($S/N=3$). Relative standard deviations of peak area and migration time for AsA in vegetables were 8.7 and 0.3%, respectively. Recovery of AsA was 97–112%. AsA concentrations in various vegetables were determined by the method. The method was also used for the determination of AsA in vegetables that had been cultivated by using fermented blue mussels, to examine their usefulness as a fertilizer for vegetables.

Keywords: Vegetables; Food analysis; Ascorbic acid

1. Introduction

Ascorbic acid (AsA) plays important physiological roles in the human body in the absorption of iron, the metabolism of steroids and aromatic amino acids, the synthesis of polysaccharides and collagens and in neonatal development [1]. It is important to determine AsA concentrations in vegetables since AsA is mainly obtained from vegetables.

AsA exists in foods in the form of AsA (reduced form) and dehydroascorbic acid (DAsA, oxidized form) [2]; both AsA and DAsA (total AsA) in foods have been generally determined by the dinitrophenylhydrazine method [3]. Nowadays the most common mode of analysis of AsA in foods, beverages or pharmaceutical products etc. is high-performance liquid chromatography (HPLC) [4] such as reversed-phase chromatography [5–9], ion-exclusion chromatography [10–14], ion-pair chromatography [15,16] or ion-exchange chromatography [17]. Flow-injection [18–21], isotachophoresis [22–25], spectrophotometry [26–28], differential-pulse polarography [29], adsorption potentiometry [30] and the method using the Landolt reaction [31] have been also used.

Recently capillary electrophoresis (CE) has been used for the determination of AsA. Micellar electrokinetic capillary chromatography (MECC) with sodium dodecyl sulfate (SDS) [32] or sodium deoxycholate [33,34] as the surfactant have been applied for the determination of AsA in a vitamin injection and the determination of total AsA in vegetables etc., respectively. Capillary zone electro-

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phoresis (CZE) methods for AsA analysis have been also developed using a coated column [35,36], uncoated column [37,38] or indirect absorbance detection [39].

In our previous paper [40], CZE using a borate buffer has been proposed for the determination of free calcium in vegetables. In the present paper, the stability of AsA during standing and during migration is investigated using CZE; although the former has been examined by several researchers the latter one has not previously been studied. The effect of hydrochloric acid on the stabilization of AsA is also investigated. Optimum analytical conditions are established for the determination of AsA in vegetables by CZE using a borate buffer (pH 9.2). Since the pK_1 and pK_2 of AsA are 4.25 and 11.57, respectively, AsA has a negative charge (−1) due to the dissociation of the C-3 hydroxyl group at pH 9.2 [41].

Blue mussels that exist on the walls of an enclosed area of the sea take up suspended organic matter from seawater [42]. These mussels can fall into the water and cause pollution during the summer to autumn period. In our previous paper [40], we proposed the collection of the blue mussels from the walls and the use of the collected mussels as a fertilizer. This will also help to prevent eutrophication of the sea. In the present study, spinach was cultivated using fermented blue mussels as the fertilizer. The concentration of AsA in the spinach was determined by the method proposed in this paper as a further example of the usefulness of this fertilizer.

2. Experimental

2.1. Apparatus

2.1.1. CZE

A Perkin-Elmer (Foster City, CA, USA) Model 270A capillary electrophoretic analyzer was used with a UV–Vis absorbance detector. The rise-time for the detector was set at 0.50 s. A polyimide-coated fused-silica capillary (GL Sciences, Tokyo, Japan), 50 μm I.D.×375 μm O.D., served as the capillary electrophoresis column. The total length of the column was 72 cm; the effective length was 50 cm. Peak area and peak height were measured using a Hitachi (Tokyo, Japan) Model D-2500 Chromato-Integrator.

2.1.2. HPLC

A Dionex (Sunnyvale, CA, USA) Model DX-AQ1211V ion chromatograph system was used with a Hitachi (Tokyo, Japan) Model L-7400 UV detector. Chromatographic separations were performed on a Hitachi 3056 column, 150 mm×4 mm I.D., and a Hitachi Model D-2500 Chromato-Integrator was also employed for integration.

2.2. Reagents

All reagents were of analytical-reagent grade and were used as received. AsA, sodium tetraborate, thiourea and metaphosphoric acid were obtained from Nacalai Tesque (Kyoto, Japan). Hydrochloric acid and glutamic acid were obtained from Wako (Osaka, Japan). Vegetables were purchased from local market stores and analyzed on the day of purchase. Distilled demineralized water was obtained from a Yamato Kagaku (Tokyo, Japan) Model WG220 automatic still and a Nihon Millipore (Tokyo, Japan) Milli-Q II system was used throughout. Standard solutions of AsA (5–50 mg/l) were prepared daily from 1000 mg/l AsA solution in 5 m*M* hydrochloric acid to prevent the oxidation of AsA.

2.3. Procedure

2.3.1. Sample preparation

Several extraction solutions have been used for the determination of AsA in foods, beverages or pharmaceutical products etc.: metaphosphoric acid [12,13,34,37], oxalic acid [20,29], trichloroacetic acid [36,37], citric acid [27], a mixture of metaphosphoric acid and acetic acid [28] and a mixture of sulfuric acid and ethylenediamine tetraacetic acid (EDTA) [10]. Metaphosphoric acid is most frequently used, but it is not easy to handle due to its rod-like shape and deliquescence. Fukuba and Tsuda [23] proposed a mixture of thiourea and hydrochloric acid as the extraction solution for AsA and showed that the extraction ability was comparable to that of metaphosphoric acid solution. In the present study,

AsA was, therefore, extracted from vegetables using a modification of the procedure proposed by Fukuba and Tsuda [23]. 2 g of chopped-up vegetables were homogenized with 4 ml of 2% thiourea–10 m*M* hydrochloric acid solution in a porcelain mortar. The whole leaf, including the leaf blade and the leaf stalk, was used for the sample. 14 ml of 10 m*M* hydrochloric acid was added. After standing for 15 min, the homogenate was transferred into a centrifuge tube with 18 ml of water which had been used for washing the porcelain mortar and the solution was centrifuged at 3000 rpm (1600 *g*) for 5 min. The supernatant solution was filtered through a 0.45-μm membrane filter. The filtrate was injected into the capillary electrophoretic analyzer and the HPLC apparatus. The concentration of hydrochloric acid in the filtrate was 5 m*M*.

2.3.2. Analysis by CZE

All solutions including a buffer solution were filtered through a 0.45-μm membrane filter before use. The detection wavelength was set at 270 nm. The thermostat was maintained at 35°C. The capillary was filled with the buffer solution (20 m*M* sodium tetraborate, pH 9.2) by vacuum for 3 min. A small amount of the sample solution (12 nl) was injected into the capillary electrophoresis apparatus by vacuum for 3 s. The volume of material injected per unit time (V_t, nl/s) is determined by the following equation [43]

$$V_t = \frac{\Delta P D^4 \pi}{128 \eta L} \quad (1)$$

where ΔP equals the pressure drop, D is the capillary internal diameter, η is the viscosity, and L is the length of the capillary. A voltage of +20 kV was applied with the sample inlet side being set as the anode. Each step was automatically run. A calibration graph was prepared by use of synthetic standards.

2.3.3. Analysis by HPLC

The following analytical conditions [44] were employed. The eluent, 1% metaphosphoric acid was prefiltered using a 0.45-μm membrane. AsA was monitored at 242 nm. The column temperature was maintained at ambient temperature. The flow-rate of the eluent was 1.0 ml/min. A 10-μl injection loop was employed.

3. Results and discussion

3.1. Stability of AsA solution

It is well known that AsA is easily oxidized to dehydroascorbic acid (DAsA) in alkaline solutions, while it is relatively stable in acidic solutions [3]. Wu et al. [39] monitored the photocatalysed oxidation of AsA under various conditions. Several solutions have been used to prevent the oxidative degradation of AsA in solutions: metaphosphoric acid [5,12], D,L-dithiothreitol [33,34], oxalic acid [20], citric acid [27], a mixture of metaphosphoric acid and acetic acid [28], EDTA [10], D,L-homocysteine [5], sulfuric acid [14], acetic acid [18], 2-mercaptoethanol [19] and perchloric acid [21]. Lloyd et al. [6] evaluated the stability of AsA in several kinds of solutions including metaphosphoric acid and D,L-homocysteine using HPLC. Schiewe et al. [38] also investigated the stability of AsA with and without the addition of L-cysteine in pH 5.0 and 8.0 phosphate buffers, respectively, using the data from UV spectra.

As mentioned in Section 2.3, the sample solution for AsA also contains 5 m*M* hydrochloric acid. We carried out the following experiment to confirm the effectiveness of hydrochloric acid in preventing the oxidation of AsA. Solutions containing 15 mg/l of AsA with and without 5 m*M* hydrochloric acid were allowed to stand at room temperature (24–25°C) in stoppered glass vessels. A portion of the solution was injected into the capillary electrophoresis apparatus every 30 min. Fig. 1 shows the variations of peak area and peak height for AsA with time. Both peak area and peak height for AsA without hydrochloric acid decreased linearly with time, but they were almost constant for at least 3.5–4 h when 5 m*M* hydrochloric acid was added. The AsA solutions containing 5 m*M* hydrochloric acid were, therefore, used as the standard solutions for AsA in all subsequent experiments.

It was possible that AsA could be decomposed in the capillary during migration under the alkaline conditions. In order to confirm this, the following experiment was carried out. The standard solution of

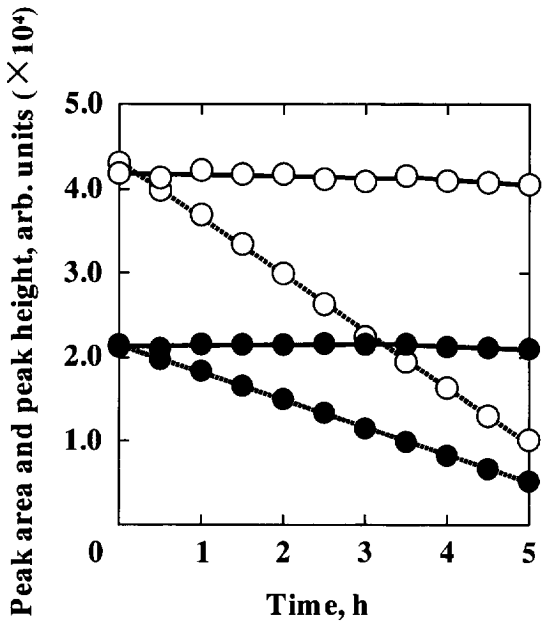

Fig. 1. Stability of ascorbic acid solutions. Solid lines: addition of 5 m*M* hydrochloric acid; dotted lines: without hydrochloric acid. ○=Peak area; ●=peak height. Sample, 15 mg/l AsA.

15 mg/l AsA containing 600 mg/l thiourea as the internal standard was analyzed using different effective length capillaries, 20–80 cm (total length, 102 cm), in order to change the migration time. The concentration of thiourea (600 mg/l) was experimentally chosen to obtain almost the same peak area as AsA (15 mg/l). The same UV transparent capillary (Supelco, Bellefonte, PA, USA), 50 μm I.D.×363 μm O.D., was used throughout to eliminate any error caused by differences in capillaries, and a fixed field strength of 28 kV was adopted. The measurements were carried out twice in each effective length. The migration time for AsA and thiourea increased linearly with the increase of the effective length. It seemed that the peak area for AsA was almost constant, whereas that for thiourea increased with effective length, as shown in Fig. 2. The regression equation relating area response (y, arbitrary units) to effective length (x, 20–80 cm) was $y=-5.77x+19\,741$ (correlation coefficient, −0.1203) for AsA. The regression equation was $y=42.3x+17\,814$ (0.6010) for thiourea. A paired t-test [45] was performed to examine the data statistically. For the pairs of mean peak areas for

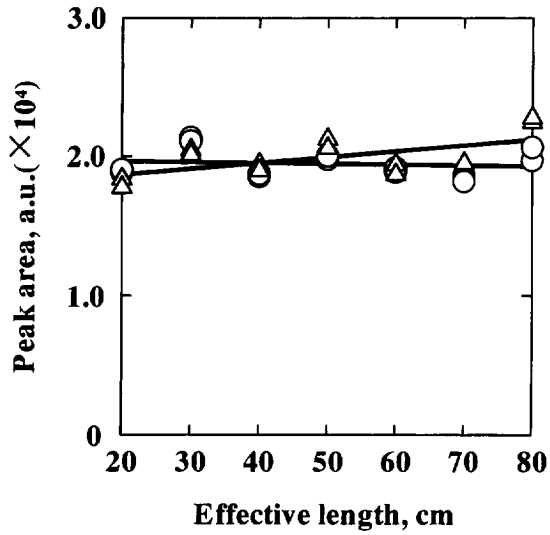

Fig. 2. Study of effective length on peak area for ascorbic acid and thiourea. ○=AsA; △=thiourea. Sample, 15 mg/l AsA containing 600 mg/l thiourea.

AsA and thiourea in each effective length, the differences were −877, −484, 583, 999, 38, 1060 and 2459. The mean difference, $\overline{x}_d$, was 540 and the standard deviation of the differences, s_d, was 1116. t is given by

$$t=\frac{\overline{x}_d\sqrt{n}}{s_d} \quad (2)$$

where n is the sample size. Substituting in Eq. (2) gives $t=1.28$. The critical value of $|t|$ is 2.45 ($P=0.05$) and since the calculated value of $|t|$ is less than this the null hypothesis is retained: AsA and thiourea do not give significantly different values for the mean peak area. It was concluded that AsA was not decomposed in the capillary during migration within 13 min (migration time for 80 cm of effective length). Tsuda and Fukuba [24] described that AsA is not oxidized in isotachophoresis even using a highly alkaline medium, as the AsA solution never comes in contact with air.

3.2. Capillary length

Assuming that band broadening is caused by only axial diffusion, the number of theoretical plates (N) in CZE is given by [46]

$$N = \frac{(\mu_{eo} + \mu_{ep})V}{2D} \cdot \frac{l}{L} \quad (3)$$

where μ_{eo} is the electroosmotic mobility, μ_{ep} is the electrophoretic mobility, D is the diffusion coefficient of the solute, V is the voltage applied across the capillary, l is the effective length and L is the total length of the capillary. If the same buffer is used, N is determined by the product of the field strength ($E=V/L$) and l. The standard solution of 15 mg/l AsA was repeatedly analyzed (8 times) using two sets of conditions where Vl/L was equal: $V_1=15$ kV, $L_1=54$ cm, $l_1=25$ cm; $V_2=20$ kV, $L_2=72$ cm, $l_2=25$ cm. The values of the relative standard deviation (R.S.D.) of peak area and peak height for AsA in the former were smaller than those in the latter, while the values of the R.S.D. of the migration time were almost equal in both conditions, as shown in Table 1. The number of theoretical plates (N) in Table 1 was calculated by the following equation proposed by Tsuda [47]

$$N = 2\pi\left(\frac{ht_R}{A}\right)^2 \quad (4)$$

where A is the peak area, h is the peak height and t_R is the retention time. The magnitude of N in the former was slightly smaller than that in the latter. The difference may be attributed to the difference of injection volume resulting from the use of the different total length capillaries.

3.3. Wavelength

Fukuba and Tsuda [23] reported that the absorption maximum of AsA was 262 nm by obtaining the absorption spectrum using spectrophotometry. In CE, 254 [33,34,36,37] or 265 nm [35] has been used for the direct detection of AsA. The effects of wavelength on the peak area and peak height for AsA were studied over the range of 260–280 nm using the conditions ($V_1=15$ kV, $L_1=54$ cm, $l_1=25$ cm). Both maximum peak area and peak height for AsA were obtained at 270 nm. Therefore, 270 nm was adopted as the optimum wavelength for the determination of AsA. The presence of glutamic acid (Glu) has been shown to interfere with the measurement of AsA in vegetables by isotachophoresis using an acidic leading electrolyte and a potential gradient detector [23], since the electrophoretic mobility of AsA was approximately equal to that of Glu in an acidic medium in studies by Fukuba and Tsuda [24]. They found that Glu had no UV absorption and solved the problem of the interference by the combined use of a potential gradient detector and a UV detector [23]. Standard solutions of AsA containing 0–150 mg/l Glu were analyzed by CZE to confirm this. Both peak area and peak height were almost constant irrespective of the concentration of Glu added, as illustrated in Fig. 3. This confirms that there was no interference from Glu for the CZE determination of AsA at 270 nm.

Table 1
Precision of determination of AsA and plate number

Total length (cm)	R.S.D. (%)			Plate number
	Area	Height	Time	
54	0.6	0.5	0.2	83 000
72	1.3	1.7	0.3	96 000

Effective length: 25 cm.
Applied voltage: 15 kV (54 cm), 20 kV (72 cm).
Sample: 15 mg/l AsA; Number of determinations: 8.

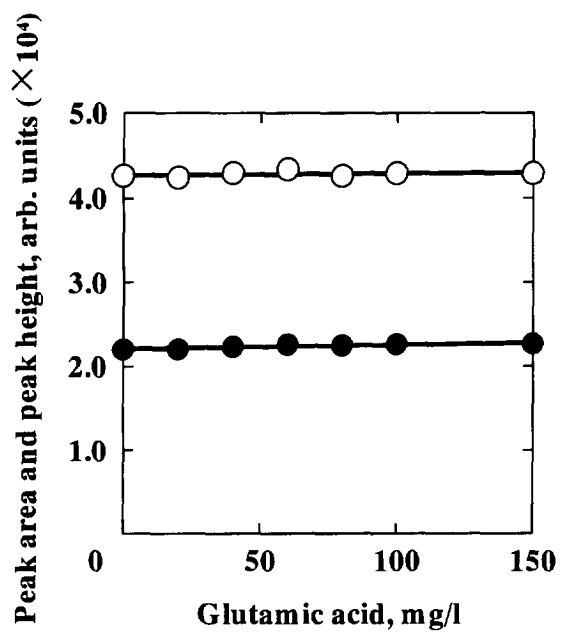

Fig. 3. Influence of glutamic acid on peak area and peak height for ascorbic acid. ○=Peak area; ●=peak height. Sample, 15 mg/l AsA containing 0–150 mg/l of Glu.

3.4. Determination of AsA in various vegetables

3.4.1. Analysis by CZE

Figs. 4A and B show the electropherograms of a spinach sample using two sets of conditions, $V_1 = 20$ kV, $L_1 = 72$ cm, $l_1 = 50$ cm, and $V_2 = 15$ kV, $L_2 = 54$ cm, $l_2 = 25$ cm, respectively. Better resolution of AsA from other coexisting components was obtained using the former conditions. According to Eq. (4), the number of theoretical plates (N) in Fig. 4A and B were 220 000 and 113 000, respectively. The 50 cm effective length capillary gave an acceptable analysis time of 9 min for AsA and was used in all subsequent experiments.

Calibration graphs for AsA were linear using both peak area and peak height up to at least 50 mg/l. The regression equation relating the area response (y, arb. units) to the concentration (x, 0–50 mg/l) was $y = 2294x - 27$ (correlation coefficient 0.9998) for AsA. The regression equation relating the height response to the concentration was $y = 745x + 79$ (0.9998). Since the magnitude of the slope of the regression line for the area response was 3 times larger than that for the height response, the peak area was used for the calculation of the concentration of AsA in vegetables. The limit of detection (LOD) for AsA was 0.35 mg/l ($S/N = 3$).

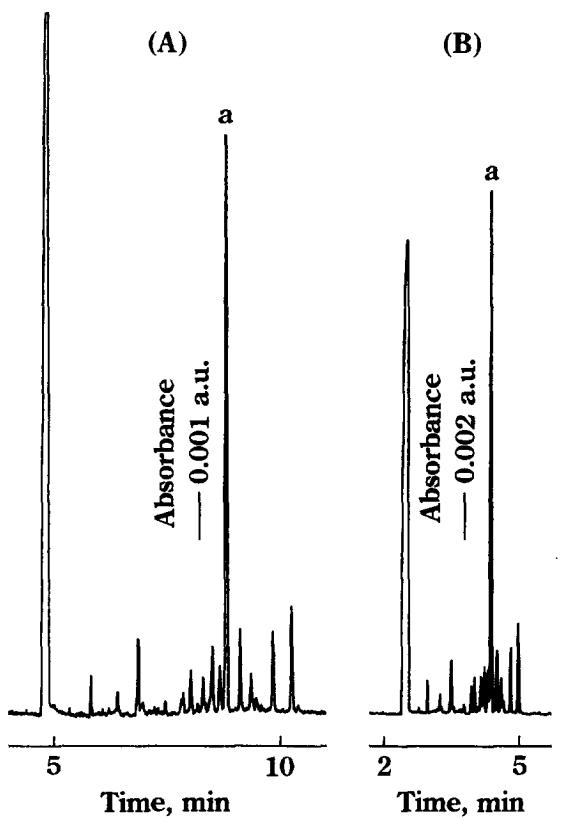

Fig. 4. Electropherograms of spinach in 20 mM sodium tetraborate, pH 9.2. Sample, purchased spinach. (A) $V_1 = 20$ kV, $L_1 = 72$ cm, $l_1 = 50$ cm; (B) $V_2 = 15$ kV, $L_2 = 54$ cm, $l_2 = 25$ cm. (a) AsA.

Table 2
Recovery of AsA in spinach

Added (mg/l)	Found (mg/l)	Recovery (%)
–	10.6	–
5.0	15.7	102
10.0	21.8	112
15.0	25.3	98
20.0	30.0	97
25.0	36.6	104

Sample: purchased spinach.

A commercially available spinach was repeatedly analyzed 4 times to examine the precision of the method. Enough spinach for 4 analyses was chopped up; 4 weighings of 2 g of the spinach served as samples and each of the 4 samples was treated by the proposed method in parallel. The values of the R.S.D. of the peak area and the migration time were 8.7 and 0.3%, respectively. Spinach samples, with 5.0–25.0 mg/l of AsA added, were analyzed by the method. The recovery of AsA was 97–112%, as shown in Table 2.

The method was used for the determination of AsA in various vegetables on the market. The results are shown in Table 3; chingentsuai and komatsuna

Table 3
Results for AsA in various vegetables

Sample	AsA (mg/100 g)
Spinach[a]	80
Spinach[b]	19–52
Chingentsuai[b]	32
Komatsuna[b]	22
Turnip[b]	72
Perilla[b]	20
Parsley[b]	97

[a] Cultivated.
[b] Purchased.

are varieties of Chinese and Japanese vegetables, respectively; 4 stumps of spinach purchased on different days were analyzed. The correlation coefficient between the concentrations of AsA in Table 3 and those of total AsA (spinach, 65; chingentsuai, 29; komatsuna, 75; turnip, 75; perilla, 55, and parsley, 200 mg/100 g) was 0.8200 (Standard Tables of Food Composition in Japan [48]).

3.4.2. Analysis by HPLC

Calibration graphs for AsA by HPLC were linear using both peak area and peak height up to at least 50 mg/l. The regression equation relating area response (y, arb. units) to concentration (x, 0–50 mg/l) for AsA was $y=4.25\times10^4x-1.90\times10^4$ (correlation coefficient 0.9997). The regression equation relating height response to concentration was $y=6.22\times10^3x-2.69\times10^3$ (correlation coefficient 0.9998). According to the procedure described in Section 2.3, AsA was extracted from a spinach sample and was analyzed by HPLC. However, AsA was not sufficiently separated from large amounts of thiourea in the sample solution; the retention time for thiourea was 1.5 min and that for AsA was 1.9 min.

3.5. Usefulness of blue mussels as fertilizer

Spinach was cultivated using fermented blue mussels as the fertilizer in a similar manner to that described in the previous paper [40]. The concentration of AsA in the cultivated spinach was determined by the method described in this paper as an example of the usefulness of this fertilizer. The AsA content in the cultivated spinach was 1.5–4 times higher than that for purchased spinach, as is evident from Table 3. This indicates the usefulness of blue mussels as a fertilizer.

4. Conclusions

The CZE method with 20 mM borate buffer solution is simple, rapid and possesses sufficient detection power, precision and freedom from interferences to be useful for the determination of AsA in vegetables. It was confirmed that blue mussels are useful as a fertilizer for vegetables.

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