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Capillary electrophoretic and extraction conditions for the analysis of *Catha edulis* FORKS active principles

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ABSTRACT

A capillary electrophoretic method, which allowed the detection and separation of the active principles of *Catha edulis*, i.e. cathinone, cathine and phenylpropanolamine, was developed. A suitable internal standard (nicotinamide), which permitted the quantification of the analytes reducing the variability of the migration times due to EOF changes, was identified. The analytical method was validated, assessing linearity, sensitivity and repeatability, showing optimal features for the analysis of the vegetable material. Moreover extraction conditions were investigated to achieve the exhaustion of the plant material in the fastest and most efficient way to meet the requirements of the Court.

1. Introduction

Catha edulis (khat), a flowering evergreen shrub or small tree belonging to the Celastraceae family, characterized by oval opposite finely toothed leaves, is native and mainly cultivated in East Africa and Arabian Peninsula [1–3]. The chewing of fresh leaves or the smoking of dried plant material is a common social and traditional habit in these countries [4]. The effect of khat assumption is mild excitation and euphoria, that is a psychoactive stimulation similar to that produced by amphetamines [5]. Khat contains three alkaloids with an amphetamine-like structure (Fig. 1): cathinone [(*S*)-(−)-α-aminopropiophenone, **1**], cathine [(*S*,*S*)-(+)-norpseudoephedrine, **2**] and phenylpropanolamine [(*R*,*S*)-(−)-norephedrine, PPA, **3**] [6,7], but the psychostimulation is predominately, or even exclusively due to cathinone, whose effect is believed to be mediated by the dopaminergic system.

Cathine is less active as a stimulant, and PPA has no psychotropic effect [8]. After harvesting, cathinone is enzimatically converted into cathine and norephedrine [6] and due to the limited shelf life of the most active component in the fresh vegetable material, the storage of the plant is crucial for the identification and quantification of this psychoactive component, which is, together with cathine, regulated as a controlled substance in many countries. In fact it was demonstrated that cathinone is stable for years in the dried khat, but it undergoes rapid decomposition in the fresh or frozen vegetable material [9]. Several analytical methods were developed for the determination of khat alkaloids either involving GC and HPLC techniques [10–14]; in the last years, the Tribunal of Busto Arsizio and Bergamo gave us the task to analyze all the materials suspected to contain khat active principles, coming from the seizures at the Malpensa and Orio al Serio airports in northern Italy. Thus, due to the high number of samples to be analyzed, we developed a fast, effective and reliable GC analytical procedure able to simultaneously detect, cathine and PPA, whose discrimination was mandatory because PPA is not a controlled psychoactive agent, exploiting a derivatization protocol which allowed an effective separation of the two components without dramatically increasing the time of analyses [15].

In this frame we were interested in the application of capillary electrophoresis, a technique, complementary to GC, endowed with similar characteristics of rapidity, selectivity and sensitivity, which is gaining importance in the forensic analysis [16] and which was applied to the separation of several amphetamine derivatives in the presence of chiral additives [17]. In this work, being not interested in chiral separations because the active principles contained in the vegetable material are enatiomerically pure, we tried to simplify the analytical method and to optimize the

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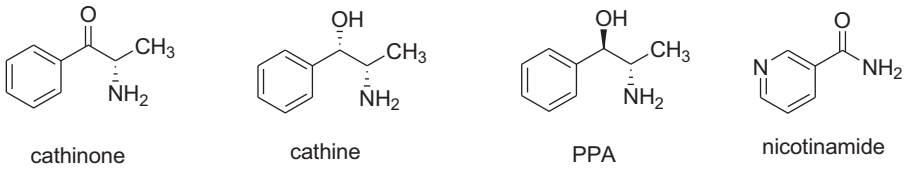

Fig. 1. Chemical structures of *S*-(−)-α-aminopropiophenone (cathinone), (*S*,*S*)-(+)-norpseudoephedrine (cathine) and (*R*,*S*)-(−)-norephedrine (phenylpropanolamine, PPA), pyridine-3-carboxamide (nicotinamide).

extraction procedure to meet the requirements of the Court. Several parameters of the extraction protocol were considered in order to find the most suitable conditions for the treatment of this perishable vegetable material; moreover the electrophoretic method was validated following the guidance on validation published by the European Medicines Agency [18].

2. Experimental

2.1. Reagents

Phosphoric acid, sodium hydroxide, trizma base, acetone, methanol, phenylpropanolamine hydrochloride, nicotinamide and ethyl acetate were obtained from Sigma–Aldrich. All reagents used were of analytical grade and all reagents, standard solutions and buffers were prepared with water obtained from a Milli-Q water purification system (Millipore).

2.2. Standards

Cathinone was purchased from LGC PROMOCHEM s.r.l., while a 1 mg/mL solution of cathine in methanol from S.A.L.A.R.S. s.p.a.

Stock solutions of 100 μg/mL of cathinone, cathine and phenylpropanolamine were prepared in methanol.

2.3. Vegetable material

The vegetable material studied (bundle 1) was seized in January 2011 and was delivered to our laboratory by the Tribunal of Busto Arsizio. It was composed by 120 bunches contained in a traveling bag. The vegetable material was completely frozen after the seizure and it was kept refrigerated until the analyses. 10 representative bundles were withdrawn from the seized material to be analyzed. Every bundle was composed of a variable number of *C. edulis* twigs divided into small groups and tied with strands of raffia. The leaves and stems of the vegetable material were wrapped with blotting paper and covered with a banana leaf.

The vegetable material from which bundle 2 was taken was seized in February 2012 and was composed of 38 bunches of *C. edulis*, the vegetable material from which bundle 3 was taken was composed of 15 bunches, seized in July 2012 in northern Italy and were delivered to our laboratory by the Tribunal of Busto Arsizio.

The plant material underwent a preliminary botanical examination in order to establish the species. From a legal point of view, therefore it was necessary to unequivocally determine and quantify the active principles.

2.4. Method development

Several parameters were taken into account in the development of the extraction protocol: solvent, method of extraction and time of contact. All the proofs were carried out starting from 1 g of dry vegetable material, which was suspended in 10 mL of extracting solvent.

2.4.1. Preparation of the vegetable material

Frozen leaves and stems were manually separated and dried at room temperature on blotting paper for 48 h. The dry vegetable material was stored up in plastic bags until use.

2.4.2. Solvent of extraction

Three different solvents were evaluated: methanol, ethyl acetate and chloroform. Leaves and stems were chopped and mixed in order to obtain a homogeneous vegetable material (1 g), which was added with the extraction solvent (10 mL). The mixture was vigorously stirred for 1 min and then macerated at room temperature. The solvent (100 μL) was withdrawn every 24 h to control the progress of the extraction. In the case of ethyl acetate and chloroform the solvent was evaporated and the residue recovered with water.

2.4.3. Method of extraction

Three different extraction methods were compared:

Maceration: 1 g of leaves and stems manually chopped in pieces of about 0.5 cm were suspended in 10 mL of solvent in a round flask, vigorously mixed for 1 min and macerated for 24 h. Every hour the solvent (100 μL) was withdrawn to control the progress of the extraction.

Extraction with a rotary extractor: the vegetable material suspended in the organic solvent was put on a rotary extractor and rotated for 7 h. Every hour the solvent (100 μL) was withdrawn to control the progress of the extraction.

Ultrasound extraction: three samples (1 g of leaves and 1 g of stems) were suspended in 10 mL of solvent. The first sample was sonicated for 20 min and then allowed to stand at room temperature for 40 min, The second was sonicated for 40 min and allowed to stand for 20 min. The third was sonicated for 60 min. After 60 min the solvent (100 μL) was withdrawn to control the progress of the extraction.

2.4.4. Time of contact

The progress of the extraction (1 g of leaves and 1 g of stems suspended in 10 mL of solvent) was monitored during the first 7 h withdrawing 100 μL of solvent, then the solvent was analyzed after 24 h and once a day for 5 days, either changing the solvent every day or without changing the solvent, in order to evaluate the time necessary to exhaust the vegetable drug. To shorten the time of extraction other two proofs were carried out (with or without changing the solvent) with 100 mg of vegetable material.

2.4.5. Evaluation of the distribution of the active principles

The vegetable material was separated into leaves, tender stems and woody stems. 1 g of the different parts of the plant were exactly weighted and suspended in 10 mL of solvent.

2.4.6. Drying of the vegetable material

The stability of the active principles was evaluated: 3 samples of frozen leaves (1 g) and 3 samples of frozen stems (1 g) were directly extracted; 3 samples of frozen leaves (1 g) and 3 samples of frozen stems (1 g) were dried at 35 °C and then extracted. In order to simplify the preparation of the vegetable material a whole

bundle of *C. edulis* was dried at 35 °C. The dried vegetable material was homogenized an extracted.

2.4.7. Optimal extraction conditions

100 mg of mixed vegetable material coming from a whole bundle (77.520 g) were macerated in methanol at room temperature (10 mL) for 48 h, changing the solvent after 24 h. Then from the combined extracts 100 μL were withdrawn and analyzed in CE (see Section 2.5).

2.5. Apparatus

Capillary electrophoretic (CE) experiments were carried out using a Beckman Coulter ProteomeLab PA 800 system equipped with a diode-array detector scanning from 190 to 600 nm. A 32 Karat software was employed for instrument control, data acquisition and data analysis. Electrophoretic separations were performed under the conventional operating conditions (anodic injection) in an uncoated fused-silica capillary of 62 cm total length, 50 cm effective length and 50 μm i.d. (Composite Metal Service Ltd.). New capillaries were preliminarily conditioned as follows: 30 min with 0.1 M NaOH, 30 min with water and 30 min with the running buffer. Between runs, the capillary was activated with 0.1 M NaOH for 3 min, rinsed with water for 3 min and with background electrolyte (BGE) for 5 min. Activation, rinse and equilibrations steps were all carried out with a pressure of 40 psi. All injections were performed in the hydrodynamic mode (20 s, 1.0 psi). The capillary was operated at 30 kV, while maintaining its temperature at 25 °C; detection was carried out with a DAD detector scanning from 190 to 400 nm. Quantitative determinations were carried out at 210 nm, since at this wavelength the samples showed their higher peak absorbance. The pH values of the running buffers were measured with a MP 220 pH meter (Mettler Toledo) equipped with an electrode InLab 418 (Mettler Toledo), daily calibrated.

25 mM TRIS phosphate buffer (pH 2.5) was prepared dissolving 605.7 mg of Trizma base in 200 mL of water and the pH was adjusted with a saturated solution of phosphoric acid 99%.

2.6. Sample preparation

When preparing the samples for the electrophoretic analysis it is mandatory that the percentage of organic solvent do not exceed 20% (v/v) of the aqueous medium. Moreover an internal standard is needed to minimize the EOF variations leading to consistent differences in the absolute electrophoretic migrations.

The samples were prepared as follows: 100 μL of extract or standard solution (0.1 mg/mL), 100 μL of nicotinamide (IS, 0.1 mg/mL) and 1.8 mL of water.

2.7. Linearity

Linearity of detector response was evaluated for each analyte preparing five standard solution at a concentration of 10, 20, 50, 100 and 200 μg/mL adding nicotinamide (0.1 mg/mL).

2.8. Sensitivity

LOD and LOQ were evaluated progressively diluting standards solutions (0.1 mg/mL) of the active principles. LOD was determined as the lowest detectable analyte concentration which was at least 3 times higher than the standard deviation of the signal to noise ratio. LOQ was determined as the lowest analyte concentration which could be quantified and was at least 10 times higher than the standard deviation of the signal to noise ratio.

2.9. Repeatabilty

Intra-day repeatability was assessed analysing the same extract six times in the same day; inter day repeatability was obtained analysing the same sample every 24 h for four consecutive days.

As regard as intra-day repeatability, relative retention times respect to the internal standard value resulted 1.038 ± 0.026 for cathinone, 1.081 ± 0.030 for cathine and 1.091 ± 0.030 for PPA, whereas relative peak areas respect to the internal standard value were 6.352 ± 0.151 for cathinone, 2.427 ± 0.127 for cathine and 0.609 ± 0.093 for PPA. As regard as inter-day repeatability, relative retention times respect to the internal standard value resulted 1.044 ± 0.023 for cathinone, 1.063 ± 0.020 for cathine and 1.108 ± 0.032 for PPA, whereas relative peak areas respect to the internal standard value were 6.238 ± 0.143 for cathinone, 2.325 ± 0.118 for cathine and 0.626 ± 0.090 for PPA.

2.10. Accuracy

Accuracy was assessed calculating the % recovery of the analytes defined as % Rec $_{\text{analyte}} = (C_{\text{exp}}/C_{\text{theo}}) \times 100$ where C_{exp} is the experimental concentration of the analyte and C_{theo} is its theoretical concentration. Accuracy was evaluated in triplicate spiking previously exhausted khat plant material at the concentration of 0.1 and 0.01 mg/mL of all the analytes. Accuracy ranged from 96.9% to 106.7% at 0.01 mg/mL and from 94.8% to 102.7% at 0.1 mg/mL for cathinone, from 98.2% to 103.2% at 0.01 mg/mL and from 96.6% to 107.0% at 0.1 mg/mL for cathine and from 98.5% to 103.3% at 0.01 mg/mL and from 97.8% to 103.1% at 0.1 mg/mL for PPA.

3. Results and discussion

For the optimization of the electrophoretic method a mixture of the standards of the active principles, i.e. cathinone, cathine and PPA, was used. Several buffers at different pH and concentrations were tested. Moreover the best operating temperature and voltage were assessed, to obtain a good resolution in a short time of analysis.

The buffer which provided the optimal separation of the peaks (6 min of analysis) was 25 mM TRIS Phosphate buffer pH 2.5, operated at 30 kV, maintaining the temperature of the capillary at 25 °C (Fig. 2). Afterwards an extract of the whole vegetable material was analyzed in the condition described to verify that there were no interference with the analytes of interest. In the electrophoretic conditions, basic substances migrates faster than neutral or acidic compounds, so it was possible to separate the three main basic active principles contained in khat from the other components of the vegetable matrix in a short time. This means that this technique is much more selective than other methods such as GC or HPLC in which more complex chromatograms are obtained [15], moreover no derivatization procedures are required. In order to quantify the active principles and to minimize the EOF variations, an internal standard (IS) was sought, whose time of migration and electrophoretic characteristics were compatible with those of the analytes of interest. Various amphetamine derivatives were investigated, but neither of them showed optimal features, interfering with the khat alkaloids. Nicotinamide was identified as suitable IS, having a migration time of about 30 s shorter than those of cathinone, the first eluting peak (Fig. 2). The IS retention time was used to calculate relative migration times of the analytes in order to increase reproducibility and the ratio between the area of the analytes and the IS area was reported to account for injection volume and dilution volume variations.

The electrophoretic method was validated in accordance with the guidelines provided by the European Medicines Agency [18].

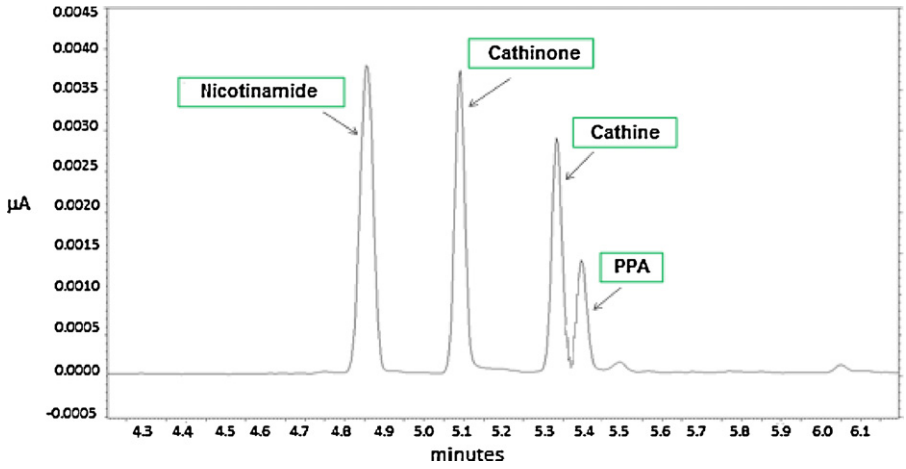

Fig. 2. Electropherogram of an extract containing the active principles of *Catha edulis* in the presence of IS (migration times: 4.292, 4.458, 4.638, 4.683).

The linearity of the DAD detector was checked for cathinone, cathine, and PPA preparing standard solution containing the IS as described in Section 2.7. The linearity of the calibration functions was satisfactory over the whole range. The calibration functions were $y = 0.0038x - 0.0281$ ($R^2 = 0.9938$) for cathinone, $y = 0.0028x - 0.0186$ ($R^2 = 0.9938$) for cathine and $y = 0.0012x - 0.0106$ ($R^2 = 0.9988$) for PPA, being y the ratio between the analyte area and the internal standard area and x the concentration of the standard solution (μg/mL).

Sensitivity was assessed diluting the standard solutions until LOQ (0.4 μg/mL) and LOD (0.2 μg/mL) were reached.

Intra-day repeatability was evaluated analysing the same extract six times in the same day; inter-day repeatability was obtained analysing the same sample once a day for four consecutive days (see Section 2.9). The %RSD values both related to the peak areas and to the migration time indicated that the method showed a suitable repeatability in the interval of time requested by the Court for the analyses. Even accuracy, obtained spiking a previously exhausted vegetable material with a known amount of the analytes, showed adequate values (see Section 2.10).

Once established the suitability of the analytical method for the determination of the alkaloids of *C. edulis*, attention was devoted to the optimization of the extraction conditions. The first issue taken into account was the extraction solvent: first of all an acidic aqueous solution was tested but the electropherogram resulted contaminated by several interfering species, then three different organic extraction media were evaluated: methanol, ethyl acetate and chloroform. From the electropherograms of the extractions carried out with these three solvents it was evident that only methanol was able to extract in an efficient way the active principles. In a previous paper [15] we reported as optimal conditions for the extraction of the analytes a liquid-liquid extraction with ethyl acetate. In this case we decided to macerate the vegetable material with pure organic solvents in order to simplify the extraction protocol and to avoid the phase separation step and the evaporation of the solvent. Methanol is particularly convenient because the extract can be diluted with the running buffer and directly injected in the instrument (see Section 2.6).

As regards as the extraction method, ultrasounds were not suitable, leading to the heating of the extraction solvent and to the decomposition of the active principles. The use of the rotary extractor did not result in improvement neither in the extraction yield nor in the extraction time. Simple maceration was therefore considered the method of choice for the extraction of khat alkaloids.

From the above mentioned proofs it was evident that it was not possible to exhaust the vegetable material (1 g in 10 mL methanol) in 24 h. Thus the progress of the extraction was monitored day by day either changing the solvent every 24 h or without changing the solvent, in order to evaluate the most effective, cheapest and fastest protocol. Leaves and stems were suspended in methanol and a little amount of the extraction solvent was withdrawn every day for 4 days. Without changing the solvent, saturation was reached in 24 h and it was not possible to exhaust the drug even prolonging the time of extraction. On the other hand, changing the solvent every day the vegatable material was completely exhausted in 4 days. In these conditions all the analytes resulted stable for all the time of the extraction-analysis protocol.

From the analyses of the active principles in the various parts of the vegetable material it could be noticed that in the leaves all the three alkaloids were detected, whereas in the stems only cathinone and cathine were present, being PPA detectable only in traces. This finding confirmed what was reported in the literature [11,15]. In the woody stems only low amounts of cathinone were found confirming the hypothesis that the most active alkaloid is enzymatically converted into cathine and PPA in the older parts of the plant (Table 1).

The drying of the vegetable material was then taken into account to confirm what was found in the study on the determination of the alkaloids of *C. edulis* by GC [15]. Three samples of frozen leaves and stems were dried in a heater at 35 °C and weighted every hour to determine the loss on drying, which

Table 1
Peak areas and relative percentages of the active principles in the various parts of the plant.

Part of the plant	Cathinone area/IS area	Cathine area/IS area	PPA area/IS area	% Cathinone	% Cathine	% PPA
Leaves	3.266	0.977	0.609	48.0	21.6	30.4
Whole stems	3.085	1.450	0	59.9	40.1	0
Woody stems	0.212	0.554	0	32.6	67.4	0
Tender stems	3.100	1.300	0	59.5	40.5	0

Table 2
Comparison between frozen and dried vegetable material. The content of khat alkaloids in the frozen material was calculated on the dry weight.

Leaves + Stems	Cathinone area/IS area	Cathine area/IS area	PPA area/IS area
Dried vegetable material	2.324	0.772	0.271
Frozen vegetable material	2.271	0.812	0.255
Mean	2.298	0.792	0.264
Standard deviation	0.037	0.028	0.011
% RSD	1.61	3.59	4.32

Table 3
Amount of active principles in bundles of *Catha edulis* coming from different seizures calculated on dry weight.

		Cathinone	Cathine	PPA
Bundle 1 (dry weight: 24.638 g)	Active principle area/IS area	0.773	0.229	0.0734
	Concentration (μg/mL)	27.7	15.0	9.4
	Total quantity in a bundle (mg)	6.82	3.70	2.32
	Quantity in a bundle (mg/g)	0.277	0.150	0.094
Bundle 2 (dry weight: 26.380 g)	Active principle area/IS area	3.176	1.120	0.251
	Concentration (μg/mL)	90.9	47.3	29.5
	Total quantity in a bundle (mg)	23.98	12.47	7.78
	Quantity in a bundle (mg/g)	0.909	0.473	0.295
Bundle 3 (dry weight: 20.810 g)	Active principle area/IS area	3.831	6.279	1.153
	Concentration (μg/mL)	108.2	234.2	104.1
	Total quantity in a bundle (mg)	22.47	48.69	21.64
	Quantity in a bundle (mg/g)	1.08	2.34	1.04

varied from 67.5 to 68.5% for the leaves and from 72.2 to 74.3% for the stems.

As a result, a sample of dried leaves and stems was extracted following the optimized extraction protocol and compared with the extraction of a sample of frozen vegetable material. Comparing the areas of the active principles it was possible to conclude that drying did not alter their composition (Table 2), confirming what previously noticed [15] and literature data [9]. These findings were further confirmed by other studies carried out on khat samples analyzed by GC–FID technique [19].

Finally to speed up as much as possible the preparation of the vegetable material and the extraction procedure in order to meet the requirements of the Court, as the active principles are present in all the parts of the plant, even if in different amounts, a whole bundle of *C. edulis* was dried and homogenized. The active principles were quantified obtaining the results reported in Table 3. In this table three bundles coming from different seizures were taken into account. The concentration of the analyte was calculated from the ratio between the area of the analyte and the area of the IS taking into account the calibration curve. The analyte concentration was transformed in the total amount of the analyte considering that 1 g of the vegetable material was macerated in 10 mL of solvent.

In bundle 1 the quantity of the active principles were very low, perhaps due to a bad conservation of the vegetable material before coming to our laboratory. In fact generally dried khat contains from 0.9 to 4.5 mg/g of cathinone [1,9–11]. In bundles 2 and 3 the content of the active principles was superior and followed in the range reported in the literature. These bundles were also analyzed by means of GC/FID technique, confirming the quantity found by CE [19].

4. Conclusions

This work takes its origin from the necessity to develop an analytical method for the determination of the alkaloids contained in the *C. edulis* vegetable material seized by the police. This point is crucial because cathine and cathinone are included in the list of the illegal substances. The characterization of the active principles must be carried out either from a qualitative or from a quantitative point of view, in fact the quantitation of cathinone and cathine is very important to discriminate from personal use and drug dealing. To this end the seized vegetable material suspected to belong to *C. edulis* species undergoes a preliminary botanic examination and then for forensic purposes the active principles must be detected and quantified. In this frame a capillary electrophoretic method was developed, allowing the detection and separation of the active principles in 6 minutes of analysis. A suitable internal standard, i.e. nicotinamide, was identified, which permitted the quantification of the analytes reducing the variability of the migration times due to EOF changes. The analytical method was validated, assessing linearity, sensitivity, repeatability, and accuracy, showing optimal features for the analysis of the vegetable material. Moreover the conservation of the vegetable material was evaluated, confirming that drying is the best way to preserve the active principles [9,15]; the extraction conditions were investigated to achieve the exhaustion of the plant material in the fastest and most efficient way to meet the requirements of the Court.

Since extracting 1 g of vegetable material, the areas of the active principles were much higher than the LOQ, to avoid solvent saturation only 100 mg of homogenized vegetable material were withdrawn and extracted in 10 mL methanol. In this way the drug was exhausted in 2 days, changing the solvent after 24 h.

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