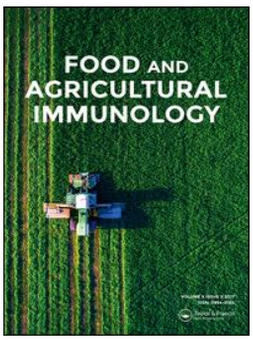

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Development of an immunochromatographic strip assay for three major capsaicinoids based on an ultrasensitive monoclonal antibody

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ABSTRACT

The problem of waste oil is a food safety issue that requires urgent resolution. In this study, a novel hapten was designed for the rapid detection of capsaicinoids, a marker of waste oil, and a monoclonal antibody (MAb) 5D7 was generated that is specific for three major capsaicinoids: capsaicin (CPC), dihydrocapsaicin (DCPC), and N-vanillylnonanamide (N-V). The half maximal inhibitory concentrations of MAb 5D7 for CPC, DCPC, and N-V were 1.12, 0.8, and 0.87 ng/mL, respectively. An immunochromatographic strip test based on MAb 5D7 was established for these three capsaicinoids, with visual inspection limits of <1.0 ng/mL, which satisfies the requirement for the rapid on-site inspection of waste oil.

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Capsaicinoids; immunochromatographic strip; monoclonal antibody; waste oil

Introduction

Waste oil has been a major quality and safety problem in the edible vegetable oil industry for many years. Because waste oil contains heavy metals, such as lead and arsenic, and other substances that are harmful to the human body (Mushak, 2003), the detection and identification of waste oil has become an issue requiring urgent resolution.

Much research into cooking oils has shown that capsaicinoids can be used as markers to detect and test kitchen waste oils because the physicochemical properties of capsaicinoids are relatively stable. Capsaicinoids are still difficult to completely remove during the process of waste oil refinement. There are more than 20 natural capsaicinoids (Schweiggert, Carle, & Schieber, 2006) and dihydrocapsaicin (DCPC) and capsaicin (CPC) combined constitute almost 90% of them (Daood et al., 2015). Scientists have synthesized

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N-vanillylnonanamide (N-V) based on the structure of capsaicinoids. Therefore, we investigated the detection of these three kinds of capsaicinoids, DCPC, CPC, and N-V.

At present, the most widely used methods for detecting capsaicinoids are high-performance liquid chromatography and chromatography–mass spectrometry (Daood et al., 2015; Liu et al., 2013; Thompson, Phinney, & Welch, 2005; Zhang, Ren, & Zhang, 2012; Zhao, Deng, Lin, Zhao, & Zhang, 2014). These methods are highly sensitive and accurate, and their results are reliable. However, they require time-consuming pretreatment of the samples, professional operators, and expensive precision instruments. Generally, immunochromatographic lateral-flow strip tests and enzyme-linked immunosorbent assays (ELISAs), both based on monoclonal antibodies (MAbs), not only display the advantages of instrumental detection technologies, but are simple to operate and meet the requirements of on-site testing in the assessment of food safety (Xing et al., 2015). Although the principle of the strip test and the ELISA are the same, insofar as both are based on an indirect competitive format, the strip test process is simpler and quicker than the ELISA, taking only a few minutes to complete. Therefore, the strip test undoubtedly better meets the need for rapid on-site detection, and the simultaneous detection of a large number of samples is possible with strips containing a highly sensitive MAb with broad specificity.

Few studies have reported MAbs or immunochromatographic lateral-flow test strips developed for the detection of capsaicinoids. Perkins et al. (2002) used an enzyme immunoassay that was developed to analyse capsaicinoids CPC and DCPC, with a limit of detection of 0.1 μg/g. Yang et al. (2016) designed four kinds of haptens based on the structure of the capsaicinoids to raise MAbs using tachyphylaxis and prepared colloidal gold immunochromatographic test strips. However, the sensitivity of the MAb for CPC, DCPC, and N-V was unsatisfactory, with half maximal inhibitory concentrations (IC_{50}) of 8.5, 5.0, and 13.5 ng/mL, respectively. Clearly, there is still a need to improve the sensitivity of MAbs directed against capsaicinoids to meet the current detection requirements. Therefore, we redesigned the structure of the hapten and used "slow immunization" in this study to generate a highly sensitive, broadly specific MAb against capsaicinoids. Based on this MAb, we developed an immunochromatographic lateral-flow strip test for the rapid detection of capsaicinoids in actual samples.

Materials and methods

Reagents and instruments

Capsaicin (CPC), DCPC, and N-vanillylnonanamide (N-V) were purchased from J&K (Shanghai, China) or Aladdin (Shanghai, China). Vanillylamine hydrochloride (VH), bovine serum albumin (BSA), ovalbumin (OVA), glutaraldehyde (GA), and goat anti-mouse immunoglobulin G (IgG) antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other solvents and reagents, including horseradish peroxidase-labeled goat anti-mouse IgG antibody and 3,3′,5,5-tetramethylbenzidine were purchased from Sigma-Aldrich. The materials (an absorbance pad, polyvinyl chloride pads, a sample pad [glass fibre membrane], and a nitrocellulose [NC] membrane) for the strip test were from JieYi Biotechnology Co., Ltd. (Shanghai, China). UV spectra were measured with an ultraviolet/visible (UV/VIS) spectrophotometer (Agilent, Santa Clara, CA), and absorbance

measurements were made with a spectrophotometric microtiter plate reader (Thermo Scientific, Waltham, MA, USA).

Synthesis of the hapten

The synthesis of the hapten is shown in Figure 1. Raw material 1 (1.0 equiv.) and raw material 2 (1.0 equiv.) were each dissolved in 3 mL of dimethylformamide (DMF), and then the solution of raw material 2 was added dropwise to the raw material 1 solution in an ice bath, and then allowed to react at room temperature. When the reaction was completed, the solution was concentrated *in vacuo* to remove the DMF. The residue was then redissolved in CH_2Cl_2. After washing with water, the organic phase was dried and compound 3 was obtained by recrystallization. Compound 3 was dissolved in trifluoroacetic acid/CH_2Cl_2 (1:1) at 0°C and concentrated *in vacuo* to produce the hapten.

Synthesis of immunogen and coating antigens

The structure of the hapten (C1) was confirmed from its 1H NMR spectrum and it was then conjugated with BSA using the GA method (Peng, Liu, Kuang, Cui, & Xu, 2017) to generate an immunogen. Briefly, 65 μL of GA (25%) was added to a solution of C1 (35.8 mg dissolved in 3 mL of DMF) with continuous stirring at room temperature. After 20 min, the reaction mixture was added to a solution of BSA (50 mg dissolved in 6 mL of carbonate buffer [0.05 mol/L, pH 9.6]) and stirred for 4 h at 4°C. The solution was then dialyzed to obtain the pure immunogen. Similarly, we used this method to conjugate hapten C1 and VH to OVA to produce two different coating antigens. The immunogen and the coating antigens were confirmed by their UV spectra, as shown in Figure 2.

Immunization process and ELISA

Immunization was performed as previously reported by our laboratory (Chen, Liu, Song, Kuang, & Xu, 2016; Peng, Liu, Xu, et al., 2017). After the immunogen was synthesized, we mixed it with the same amount of adjuvant and used it to immunize mice. For the first

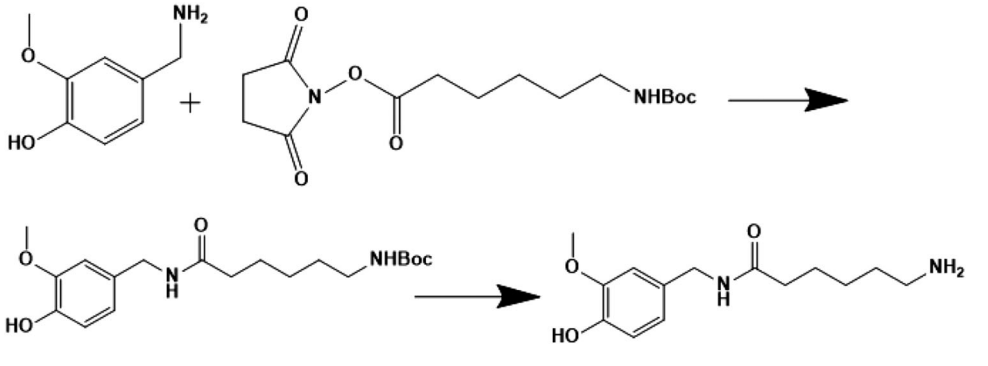

Figure 1. Synthesis of hapten C1.

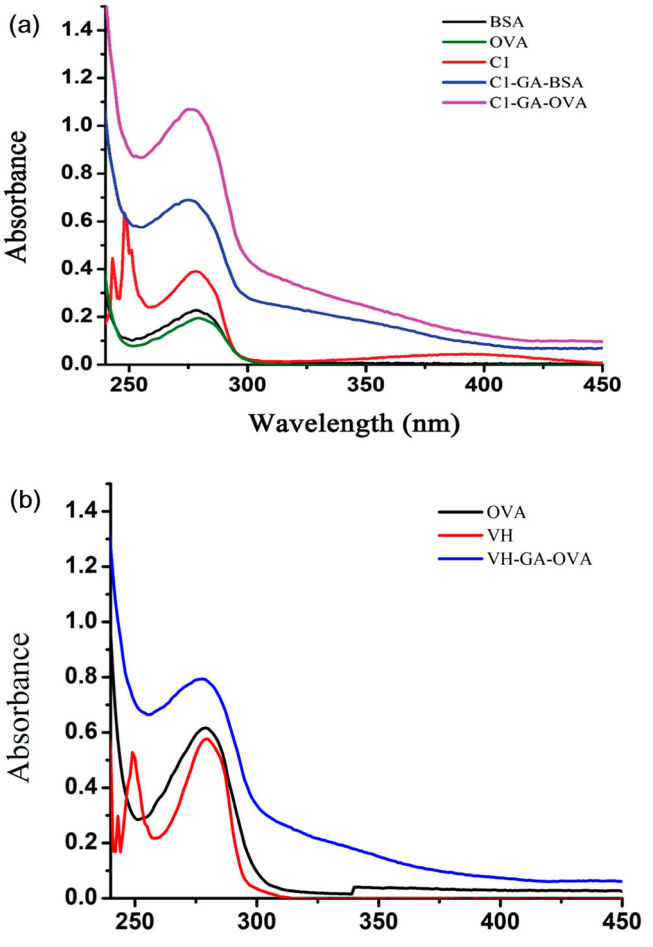

Figure 2. UV/VIS spectra of hapten C1 and hapten C1-protein conjugation.

immunization, complete adjuvant was mixed with the immunogen (final concentration 1 mg/mL), and the injection dose was 100 μg of immunogen. For the second immunization, incomplete adjuvant was added to the immunogen and the dose was halved. After the mice were immunized three times, we obtained their serum by sampling their tail blood. The sera were then screened with the two different coating antigens using an indirect competitive ELISA (ic-ELISA), and we identified the appropriate coating antigen at the same time. After the sixth immunization, the mice with antibody high titres and good inhibition rates were identified and their spleens used for cell fusion. Three days before cell fusion, 25 μg of immunogen directly dissolved in 100 mL of saline were injected into the abdomen of the mice.

ic-ELISA can be used to effectively measure the sensitivity and cross-reactivity (CR) of a MAb. The appropriate concentrations of the MAb and coating antigens were determined with reference to the literature (Peng, Liu, Xu, et al., 2017; Wang et al., 2016). After these were determined, the IC_{50} (concentration of the compound that produced 50% inhibition of antibody binding to the coating antigen) was calculated, as an important indicator of the sensitivity of the MAb. Based on these data, we determined the IC_{50} values of the three

capsaicinoids and used the following equation to calculate its CR, the criterion of MAb specificity.

$$CR\% = (IC_{50}\text{value of DCPC})/(IC_{50}\text{value of related compound}) \times 100.$$

Cell fusion and screening hybridoma cells

The processes of cell fusion and hybridoma screening were performed with methods commonly used in our laboratory (Chen et al., 2015; Song et al., 2016; C. Sun, Liu, Song, Kuang, & Xu, 2016). After three rounds of subcloning, several good hybridoma cell lines were isolated. The cell lines were cultured on a large scale and were injected into the abdomen of mice. About seven days later, the ascites produced by the corresponding cell lines were extracted and purified. After dialysis, the MAbs were obtained and their concentrations determined with UV/VIS spectroscopy at 278 nm.

Immunochromatographic lateral-flow strip test

Preparation of colloidal gold

Gold nanoparticles were synthesized with the trisodium citrate reduction method, as follows. $AuCl_3{\cdot}HCl{\cdot}4H_2O$ solution (100 mL) was boiled with constant stirring, and 2 mL of freshly prepared trisodium citrate (1%, w/v) was added with constant stirring. After 1 min, the color of the solution changed to crimson, and the solution was boiled for 15 min. The volume of the solution was then adjusted to 100 mL with ultrapure water, cooled to room temperature, and stored at 4 °C until use. The gold nanoparticles were characterized with a UV/VIS spectrophotometer and transmission electron microscopy. The maximum UV absorption peak occurred at a wavelength of 520 nm and the average diameter of the nanoparticles was 20 nm.

Preparation of colloidal gold–MAb conjugate

The principle upon which this reaction is based is that negatively charged colloidal gold can be combined with a positively charged MAb through electrostatic interactions. First, 1 mL of colloidal gold solution was added to an appropriate amount of K_2CO_3 solution (0.1 M) to adjust the pH to 8 (Guo et al., 2015; Sun et al., 2012). Then the MAb was added to the colloidal gold solution, and the reaction was vortexed for 2 h at room temperature. A 10% solution of BSA in 100 μL of ultrapure water was added to seal the unbound sites on the colloidal gold to avoid the non-specific adsorption to the gold nanoparticles. After the reaction had proceeded for 2 h, the solution was centrifuged at 875*g* for 25 min to separate the sediment. After it was washed with ultrapure water, the final precipitate was dissolved in 0.1 mL of borate buffer (0.002 M, pH 8, containing 1% (w/v) sucrose and 0.01% Tween-20), which was stored at 4°C.

Principle of the strip test

The principle of the colloidal gold immunochromatographic assay for capsaicinoids is based on an immune competition model similar to that of ic-ELISA. The detection limit T-line was established by immobilizing the capsaicinoid detection antigen on the NC membrane, and the goat anti-mouse IgG antibody was fixed to the membrane in

the control line (C-line). The test strips were then inserted into the sample extract and the end of the sample pad rapidly wetted. The colloidal gold–MAb immobilized on the conjugate pad dissolved and started to flow with the sample to the NC membrane by capillarity. With negative samples, the colloidal gold–MAb binds to both the coating antigen on the T-line and goat anti-mouse IgG antibody on the C-line, producing two visible red lines. In contrast, with positive samples, the colloidal gold–MAb binds to the capsaicinoids in the sample and cannot therefore completely combine with the coating antigen on the T-line, resulting in a T-line that is either light or disappears. Therefore, the capsaicinoid content of the sample solution can be determined according to the intensity of the colour of the T-line. The higher the free capsaicinoid content in the sample, the lighter the T-line colour will be (Chen et al., 2016; Chen et al., 2017).

Recovery test

Standard solutions of CPC, DCPC, and N-V at different concentrations (5, 10, and 20 ng/mL) were added to capsaicin-negative edible oil samples provided by Jiangsu Entry-Exit Inspection and Quarantine Bureau to prepare differently spiked oil solutions. A volume of 40 mL of methanol solution was added to 5 g of each spiked oil solution, and vortexed for 10 min, and the organic layer was separated with refrigerated centrifugation. A part of the organic layer (4 mL) was then dried with a stream of nitrogen gas and reconstituted with 5 mL of 10% methanol in 0.01 M phosphate-buffered saline (PBS). The final concentrations of the three capsaicinoids were 0.5, 1, and 2 ng/mL and analysed with an ELISA. Each experiment was repeated three times.

Results and discussion

Hapten

The accurate deign of a hapten is essential for the generation of a good MAb. Moreover, different hapten structures produce MAbs with different CR. Based on the common structure of the three major capsaicinoids and the immunogenic effects of the four different haptens designed by Yang et al. (2016), we confirmed that the exposed phenolic hydroxyl group, benzene ring, and oxymethyl group on the molecule are important to the specificity of capsaicinoid identification and cannot be altered. Therefore, in this study, we redesigned the hapten to be used as an immunogen. The raw material 1 we selected has specific structures shared by three kinds of capsaicinoids, namely phenolic hydroxyl group, benzene ring, and oxymethyl group. After reacted with the raw material 2, these specific structures were retained, and one of the original amino positions was introduced a 6-carbon long chain. Therefore, compared to the hapten C reported by Yang et al. (2016), the connecting arm of hapten C1 was lengthened. After hapten C1 coupling of carrier proteins, all functional groups in three capsaicinoids structures were retained to the utmost extent. Hapten C1 showed very good inhibition of the three capsaicinoids tested.

Screening antisera

Using an ic-ELISA, the mouse sera obtained after immunization with the synthesized immunogen described above were simultaneously tested with homologous and

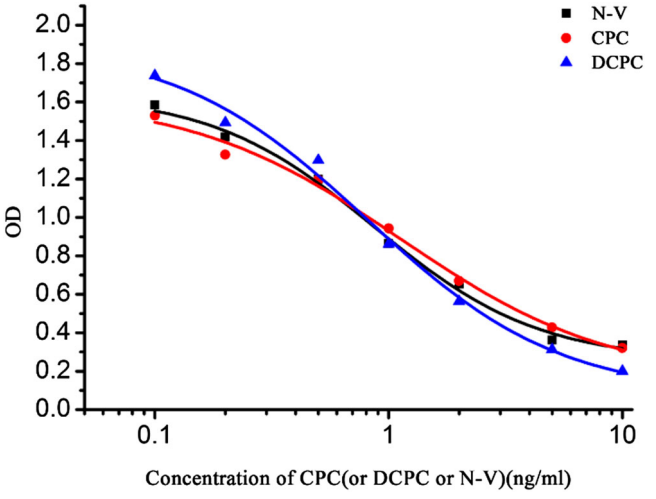

Figure 3. The IC_{50} value of the Mab 5D7 for three capsaicinoids (CPC, DCPC, and N-V).

heterologous coating antigens. For heterologous coating antigen, the potency was too low to judge the inhibition. Therefore, we determined that the homologous coating happen C1–GA–OVA was the appropriate antigen in terms of both its potency and its inhibitory effect. The IC_{50} values of MAb 5D7 for the three capsaicinoids are shown in Figure 3.

CR of MAb 5D7

The CR of MAb 5D7 is shown in Table 1. The common structures of the three major capsaicinoids were fully exposed and the tethers were lengthened when we designed the hapten. Therefore, the CR and IC_{50} values of MAb 5D7 against the three major capsaicinoids were all satisfactory. The CR data for MAb YQQD8 reported by Young et al. are also listed in Table 1. The sensitivity and CR of MAb 5D7 are clearly superior to those of MAb YQQD8.

Recovery test

Most waste oils are characteristically highly viscous, with complex components. Therefore, during the pretreatment of the samples, we referred to the study by Zhang et al., (2012) and used a liquid–liquid extraction method to extract the capsaicinoids from the samples. After proper dilution, all matrix interference was eliminated. As shown in Table 2, the average recovery of capsaicinoids from the edible vegetable oil samples was

Table 1. The IC50 and CR of three capsaicinoids of Mab 5D7 and Mab YQQD8 by icELISA.

	5D7		YQQD8	
Compounds	IC50(μg/L)	CR(100%)	IC50(μg/L)	CR(100%)
CPC	1.12	71.4.	8.5	58.82
DCPC	0.8	100	5	100
N-V	0.87	91.95	13.5	37.04

Table 2. Recoveries of capsaicinoids in edible vegetable oils samples by icELISA.

Compounds	Spiked (ng/mL)	Mean(ng/mL) ± SD	Recovery (%)	CV (%) ($n = 3$)
CPC	0.5	0.44 ± 0.08	88	4.7
	1	0.85 ± 0.05	85	5.2
	2	1.75 ± 0.14	87.5	3.8
DCPC	0.5	0.41 ± 0.09	82	5.3
	1	0.84 ± 0.13	84	2.7
	2	1.72 ± 0.08	86	4.3
N-V	0.5	0.43 ± 0.11	86	4.0
	1	0.83 ± 0.02	83	3.1
	2	1.69 ± 0.15	84.5	4.8

82–88%, indicating that this pretreatment method was effective for the detection of capsaicinoids in oil samples.

Immunochromatographic assay development

Different concentrations of CPC were added to PBS and were tested with the strip assay to assess its sensitivity. As shown in Figure 4, when there was no target present, both the T-line and the C-line were red, indicating that the colloidal gold–MAb had reacted with the immobilized antigen and the goat anti-mouse IgG antibody. When the concentration of CPC was 2 ng/mL, only the C-line was observed, so this concentration was defined as the cut-off value in evaluating the sensitivity of the strip.

Immunochromatographic strip assay

To validate the strip test method developed here, the three capsaicinoids were incorporated into negative edible oil samples, as described above. Different concentrations of CPC, DCPC, and N-V were prepared in these samples after they were pretreated as described above. The results are shown in Figure 5. A deep red colour was observed on the T-line with the naked eye when 0 ng/mL capsaicinoid was added. When the concentrations of DCPC and N-V were 1 ng/mL in the samples, the T-line was completely eliminated. When the concentration of CPC was 1 ng/mL, the T-line became significantly paler than that produced by the solution containing no capsaicin. When the concentration of

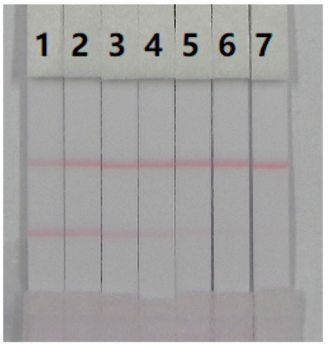

Figure 4. Image of the detection of a series concentration of standard in PBS. CPC: 1 = 0 ng/mL, 2 = 0.05 ng/mL, 3 = 0.1 ng/mL, 4 = 0.2 ng/mL, 5 = 0.5 ng/mL, 6 = 1 ng/mL, 7 = 2 ng/mL.

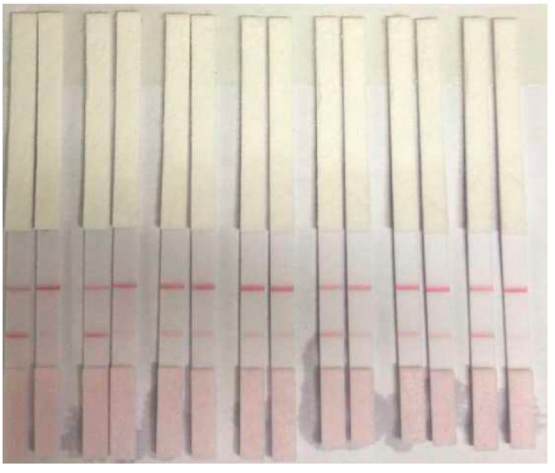

Figure 5. Images of DCPC, N-V, and CPC detection using the immunochromatographic strip assay in pretreatment negative edible oil samples. The concentrations of DCPC were 0 and 1 ng/mL (1 = 0 ng/mL, 2= 1 ng/mL). N-V (1 = 0 ng/mL, 2 = 1 ng/mL). CPC (1 = 0 ng/mL, 2 = 1 ng/mL, 3 = 0 ng/mL, 4 = 2 ng/mL).

CPC was 2 ng/mL, the T-line disappeared completely. Therefore, the cut-off value for the test strip in actual oil samples was <2 ng/mL, which meets the requirements for the rapid on-site detection of capsaicinoids.

Conclusions

In this study, MAb 5D7, which detects three major capsaicinoids with high sensitivity (IC_{50} 0.8–1.12), was prepared using the novel hapten C1. The sensitivity of MAb 5D7 was nearly 10 times higher than that of other MAbs reported in the literature (Yang et al., 2016), so it is the most sensitive MAb for capsaicinoids so far recorded. Based on this MAb, we developed an immunochromatographic strip assay for the detection of capsaicinoids in edible oil samples. The cut-off values for the colloidal gold test strip for three major capsaicinoids (DCPC, N-V, and CPC) were 1.0, 1.0, and 2.0 ng/mL, respectively, confirming that this method can be used for the rapid detection of waste oils.

Disclosure statement

No potential conflict of interest was reported by the authors.

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