# Detection of Irradiation of Meats by HPLC Determination for *o*-Tyrosine Using Novel LASER Fluorometric Detection with Automatic Pre-column Reaction

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(Received February 14, 2000; Accepted May 9, 2000)

**An *o*-Tyrosine method for detection of irradiation of foods was studied by HPLC using a novel light amplification by stimulated emission of radiation (LASER) fluorometric detection system with pre-column reaction. Sample was prepared and purified by eliminating fat and sugars using a mixture of acetone and chloroform, and then the purified protein was hydrolyzed using hydrochloric acid at 110°C for 24 h in a vacuum. The sample was reacted with 4-fluoro-7-nitrobenzofurazan (NBD-F) reagent by an automatic pipetting system and was introduced into the HPLC system. Irradiated chicken, pork, beef, and tuna were examined by irradiating at 0, 1, 5, 10 kGy. Irradiation of chicken and pork irradiated at or over 10 kGy was successfully detected, but that of beef and tuna were more difficult to detect. After 3 months storage at – 20°C, the irradiation was still detectable in chicken irradiated at 10 kGy. Thus this detection procedure can be used to detect irradiation in some chilled meats irradiated at 10 kGy. Non-irradiated *o*-tyrosine formation and reduction of *o*-tyrosine by hydroxylation are also discussed.**

**Key words** —— *o*-tyrosine method, irradiated food detection, NBD-F, HPLC, pre-column derivatization

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## INTRODUCTION

Adequate methods for detection of irradiation of foods have not yet been established, and most of the previously proposed methods have suffered from limited applicability and poor reproducibility. Although both physical methods, such as ESR and thermoluminescence (TLD) methods, and chemical methods, such as the hydrocarbon detection method (HC) and cyclobutanones method (CB), have been validated by the British Standards Institute, those methods are qualitative, rather than quantitative identification procedures.

The *o*-tyrosine method is based on oxidation of phenylalanine residues of protein in food by hydroxyl radicals that are produced by irradiation (Fig. 1). Several studies have shown that this procedure is useful for detection of irradiation of foods.[1,2] It was demonstrated that this chemical reaction is affected by temperature, oxygen pressure, and absorbed dose.[3,4]

There are conflicting results in regard to the presence of *o*-tyrosine in non-irradiated intact system; with some researchers detecting it,[5,6] and others not.[7] Several researchers have reported that *o*-tyrosine was produced during the pretreatment process in analysis. These studies suggested that the cause of the *o*-tyrosine formation may have been mitochondria in food or the extraction solvent.[8,9]

However, the *o*-tyrosine method has remarkably wide application to a good range of foods compared to other chemical and physical methods.

The novel light amplification by stimulated emission of radiation (LASER) detection system of HPLC is useful for detection of 4-fluoro-7-nitrobenzofurazan (NBD-F) derived *o*-tyrosine. Combination of automated pre-column derivatization with the LASER detector and octadecyl silica (ODS)-HPLC creates a highly powerful tool for *o*-tyrosine detection in irradiated samples.

In this study, we re-evaluate the efficacy of the *o*-tyrosine procedure using the sensitive *o*-tyrosine detection system describe above.

## MATERIAL AND METHODS

**Apparatus ——**

*Irradiation Apparatus*: The wet type $^{60}$Co plate source irradiation apparatus and table type $^{60}$Co rod source were used at 6 kGy/h and 1.0–10 kGy/h, respectively. Samples (5 g) in Pyrex test tubes were

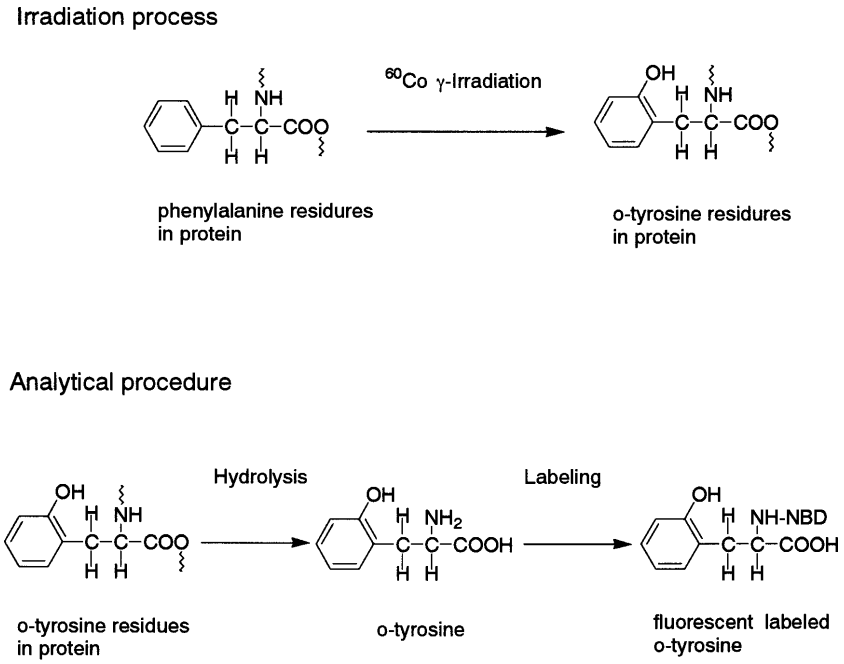


**Fig. 1.** Schematic Reaction Chart

irradiated at room temperature.

*HPLC System*: The detailed analytical instrument system has been described previously (Miyahara *et al.*, submitted), as well as has the pre-column HPLC system used for *o*-tyrosine derivatization (Miyahara *et al.*)[3] Accordingly, here we will provide only brief explanations of these systems. The instrument system consisted of a Shimadzu model LC10A pump system for high performance liquid chromatography and a Tosoh model LA8010 LASER fluorescence detector, which was operated at 488 nm for excitation and 550 nm for emission, an auto sampler (Shimadzu model SIL10A) *etc*. An ODS column (Shimadzu ODS-2; 250 mm × 4.7 mm; i.d., 5 mm; Shimadzu, Co. Kyoto, Japan) was used. The column oven was maintained isothermally at 45°C. Injection was performed by an auto-sampler with a completely filled 20-$\mu$l injection loop. Data were processed with a Shimadzu model CR7A data processor.

*Derivatization*: The derivatization conditions consisted of mixing 20 $\mu$l of sample, 10 $\mu$l of saturated borate, and 10 $\mu$l of NBD-F acetonitrile solution, incubating for 10 min, adding 0.1 N hydrochloric acid, and mixing twice. This derivatization procedure was performed automatically in the auto-sampler.

*Homogenize*: The homogenize was a Poltroon model BT1020 350B with 12 mm cutter, Kinematical, AG, Luzon.

*Vacuum Hydrolysis Tube*: The vacuum hydrolysis tube was 20 ml tube, Kontes, Vineland.

**Reagents and Other Materials** —— Amino acid standards, mobile phase for HPLC and other reagents for analysis are described in our previous paper.

**Mobile Phase for HPLC** —— Solvent A was consisted 5 mM sodium phosphate buffer at pH 6.87. Solvent B was 80% aqueous acetonitrile. A gradient mobile phase of solvents A and B was programmed as follows. The percentage of solution B was maintained at 30% for 20 min, then was increased linearly to 40% at 0.5%/min from 20 min to 40 min, and finally was maintained at 100% from 40 min to 60 min. The flow rate was 1 ml/min.

**Standard Solutions** —— Standard solution consisted of 100 mg of each amino acid dissolved in 100 ml of water.

**Sample** —— Samples for irradiation were obtained from retail markets in Setagaya-ku, Tokyo.

**Irradiation Procedure** —— Five-gram samples of meat were placed in 10 ml Pyrex tubes with stoppers. Samples were irradiated at 6 kGy/h with a plate-type source for precision irradiation. Mainly, samples were irradiated with a rod-type source at the appropriate dose rate. After irradiation, the samples were stored at – 20°C.

**Dosimetry** —— Absorbed doses were measured with a CPI (Chemical Process Indicators, NAS, OH)

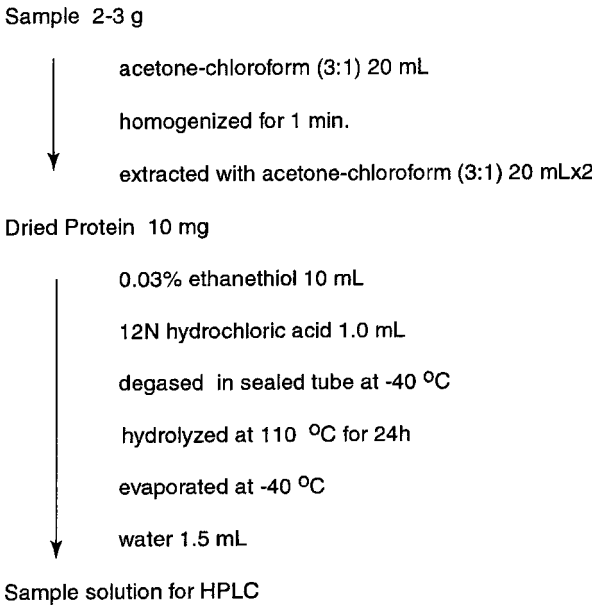


**Fig. 2.** Flow Chart for Sample Preparation

and a Radix RN-15 (Radie Kogyou, Takasaki, Japan). All dosimetric measurements were performed at room temperature.

**Sample preparation** —— Irradiated samples (2–3 g) were homogenized with a 20 ml mixture of acetone–chloroform (3 : 1) for 1 min. The mixture was then filtered and the homogenation procedure repeated. The residue on the filter was dried in a vacuum. The dried protein (10–20 mg) was placed under vacuum hydrolysis tube and was hydrolyzed by hydrochloric acid (2 ml) with 0.03% ethanethiol at 110°C for 24 h in a vacuum after purging dissolved oxygen in the sample solution by repeated cycles of pressure-reduction at – 40°C and room temperature. After the reaction mixture was cooled down to room temperature, the hydrochloric acid was evaporated off. The residue was dissolved in 1.5 ml water or borate buffer.

**Caution** —— The gamma-irradiation instrument should be operated under careful monitoring and under the supervision of a well trained professional. NBD-F and some of the organic solvents used in this study are suspected carcinogens. Handle them with care.

## RESULTS

### Analytical Conditions

The sample solutions were prepared by the USDA method[10] with slight modification. As shown in Fig. 2, the modified procedure consisted of protein-extraction from the sample followed by hydrolysis using hydrochloric acid and ethanethiol, which was substituted for thioglucolic acid as the anti-oxidant. The weight of isolated protein for hydrolysis was between 10 mg and 7 mg. Recovery test was conducted spiking 1 $\mu$g *o*-tyrosine to 10 mg chicken protein. Mean of recoveries of three trials was 81% and RSD (relative standard deviation: statistical meaning of the word is percentage of standard deviation to mean) was 12%. The original procedure has been validated by USDA (recovery, > 90%, RSD, < 5%).[10] The difference between the present and the previously published data can be explained by the difference of detectable concentration. That is, the food safety and inspetion service (FSIS) standard method was designed for the samples containing 3.5% tyrosine. On the other hand, this procedure was for samples containing less than 60 ppm *o*-tyrosine.

### Dose Response and Background Levels

To elucidate the efficacy of the *o*-tyrosine procedure and to demonstrate that gamma irradiation generates *o*-tyrosine in food, samples of chicken, pork, beef, and tuna were irradiated at 0°C to 10 kGy. As shown in Figs. 3 and 4, non-irradiated samples showed a small *o*-tyrosine peak. The background levels were 23, 6, 4, 2 $\mu$g/g for chicken, pork, beef, and tuna, respectively. Ten samples for each food, a total 40 samples, were analyzed to obtain the back ground levels in chicken, pork, beef, and tuna. These background levels were comparable with those previously reported.[1] At 10 kGy, the concentrations of *o*-tyrosine differed among the samples (for chicken, the level was 47 $\mu$g/ml). The RSD for chicken, pork, beef, and tuna, were 3.9–4.8, 4.1–5.1, 3.8–5.4, and 3.7%, respectively. However, the phenylalanine contents did not vary as widely among samples. We currently have no explanation for this difference in variability between *o*-tyrosine and phenylalanine.

As shown in Fig. 4, the dose responses range from 0 to 10 kGy. The values shown in the figure were means of triplicate measurements at each dose. The increase in *o*-tyrosine level at 10 kGy was 20–30%. Based on the results, this method can identify irradiation of chicken and pork at 5 kGy or higher.

### Possibility of *o*-Tyrosine Formations in Non-Irradiated Food

Several authors have postulated that *o*-tyrosine forms under certain specific conditions such as in the presence of organic solvents, hydrochloric acid,

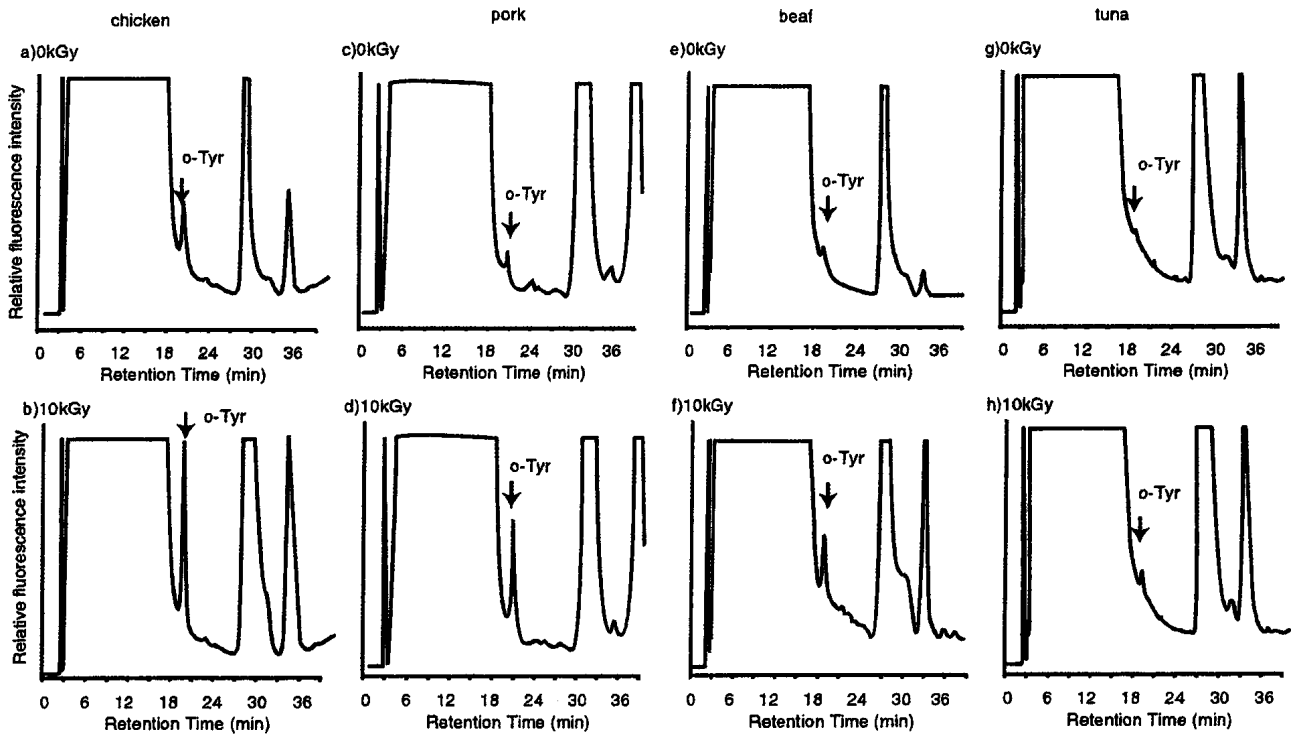


**Fig. 3.** Chromatograms of Non-Irradiated Chicken (a), Pork (c), Beef (e), Tuna (g), and 10 kGy Irradiated Chicken (b), Pork (d), Beef (f), Tuna (h)

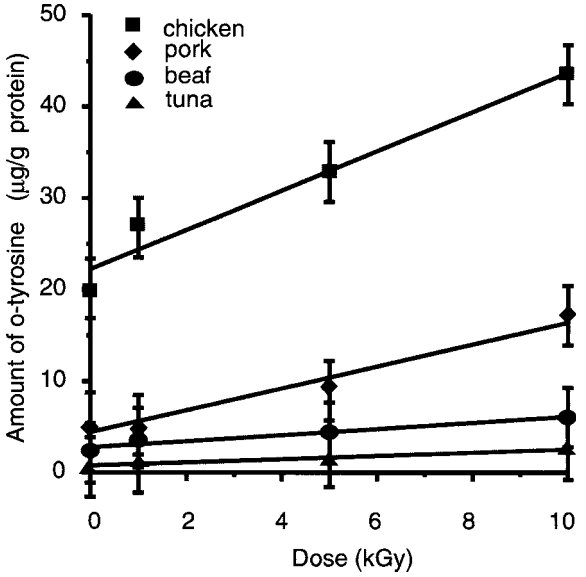


**Fig. 4.** Dose Response Curve of 4 Foods

and tissue homogenates.[8] To confirm these findings, we conducted several experiments.

Formation of *o*-tyrosine from phenylalanine in organic solvents was examined by refluxing phenylalanine (1 mg/ml suspension) with carbon tetrachloride, chloroform, and acetone for 24 h. The solvents were evaporated and residues were dissolved in water prior to measurement. No *o*-tyrosine was found in the residues, as shown in Fig. 5a, indicating that chlorinated carbon does not produce any *o*-tyrosine under these conditions.

Next we examined the formation of *o*-tyrosine by hydrolysis for 24 h with 12 N hydrochloric acid. Phenylalanine (1 mg/ml) was heated at 110°C for 24 h and the hydrochloric acid was evaporated. The residue was dissolved in water for analysis. A trace amount of *o*-tyrosine (> 0.5 ng/ml) was detected in the supernatants (Fig. 5b).

Formation of *o*-tyrosine by mitochondria/p-450 was examined by incubating phenylalanine in 50% bovine liver homogenate for 30 min. The reaction mixture was centrifuged and the supernatant was subjected to analysis. A trace amount of *o*-tyrosine was observed, and a significant amount of *p*-tyrosine was not formed (Fig. 5c). The peaks in the UV spectra of this sample, which ranged from 200–700 nm, were identical to those of the authentic *o*-tyrosine.

These results indicate that the analytical procedure does not produce any significant amount of *o*-tyrosine from phenylalanine. This is adequate for the determination of *o*-tyrosine in food.

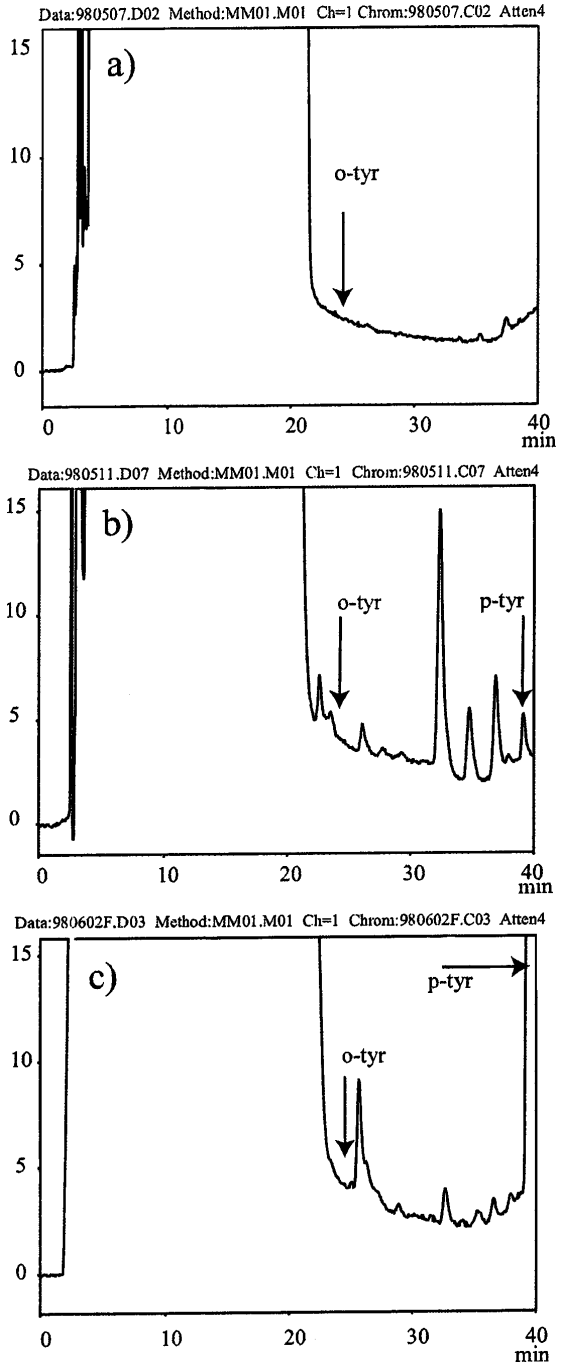


**Fig. 5.** Chromatograms of the Residue of the Refluxed Phenylalanine with Carbon Tetrachloride (a), with 12 N Hydrochloric Acid for 24 h (b), and the Residue of the Incubated Phenylalanine in 50% Bovine Liver Homogenate for 30 min (c)

## DISCUSSION

Because, ideally, this procedure would be applied to routine laboratory work, many additional factors will need to be examined. A technical and practical criteria for irradiated food detection procedure are presented by the committee for ADMIT (Analytical Detection Methods for Irradiation Treatment).[11] We evaluate this method based on those criteria.

### Discrimination

In the present study, the some pretreatments investigated produced trace amounts of *o*-tyrosine. Some background level in samples was also observed. The background levels in chicken and pork were significantly smaller than the levels of *o*-tyrosine produced by irradiation. Therefore, this method can detect irradiation of foods.

### Specificity

Our study showed that *o*-tyrosine was not induced by storage or the analytical processes used in this study.

### Applicability

This procedure can be applied throughout the dose range relevant to practical irradiation (> 5 kGy).

### Stability

After 3 months, irradiation of foods could still be identified.

### Robustness

Results were affected by dose rate (absorbed dose per unit time), temperature and admixture with other foods.[3]

### Independence

The procedure is effective for every type of meat sampled, independent of the original sample and does not require simultaneous use of paired non-irradiated samples when used for qualitative identification.

### Reproducibility and Repeatability

The results obtained in this study were repeatable. Reproducibility was not tested.

### Sensitivity

The procedure can detect irradiation of most foods that were irradiated at or over 5 kGy at room temperature, but the detectable dose is dependent on the type of sample as discussed above.

### Dose Dependence

The procedure is dose dependent. The production is proportional to the absorbed dose (absorbed

dose means how much dose or energy that the sample absorbed under specific conditions; discriminating "irradiated dose"). But dose determination at low levels is difficult due to background levels. The procedure should be capable of detecting absorbed dose, in order to identify non-irradiation of foods and overdose samples for enforcement purposes. The *o*-tyrosine procedure can be utilized for such purposes.

**Simplicity**

The procedure includes standard amino acid analysis processes. The method of pretreatment of samples has been well established by the USDA FSIS for many years.[10] Derivatization is automated and is a very simple and easy process.

**Low Cost**

The total cost for the identification is relatively low. Most of the necessary instruments are already available in most chemical laboratories with the exception of the LASER fluorometric detector.

**Speed**

The procedure requires about 27.5 h (30 min for purification, 24 h for hydrolysis, 1 h for evaporation and 2 h for HPLC analysis).

**Applicability**

Most foods contain phenylalanine as a precursor of *o*-tyrosine and thus the procedure can be applied to most samples which are requiring irradiation. In our study, 4 representative types of meat were examined. Irradiation of pork and chicken at 10 kGy was successfully identified. However, irradiation of beef and tuna are difficult to identify because of the relatively high backgrounds and low yields of *o*-tyrosine in irradiated samples. But these problems will be solved by appropriate modifications in further study.

**Non Destructiveness, Easy Standardization, and Cross Calibration**

This procedure is destructive and easily standardized. But cross calibration has not been examined.

**Resistivity to Fraud**

*o*-Tyrosine is not resistant to fraud. When an irradiated sample is diluted with a large volume of non-irradiated meat, it is difficult to identify irradiated sample.

In conclusion, the *o*-tyrosine detection method is comparable to other established methods. The recovery test, the dose response study, the back ground level survey, and the stability test illustrated that this detection procedure is adequate to detect irradiated chicken and pork. The detectability of overdose samples as well as CB is outstanding compared with those of other methods, because the dose response curve can be extended over 50 kGy. Thus, this procedure is useful for the purposes of labeling compliance when used in conjunction with established physical methods.

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