Determination of *o*-Tyrosine Production in Aqueous Solutions of Phenylalanine Irradiated with Gamma Ray, Using High Performance Liquid Chromatography with Automated Pre-column Derivatization and LASER Fluorometric Detection

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Tyrosine isomers produced by gamma radiation of aqueous phenylalanine solutions at mid dose levels (1—10 kGy) were examined to obtain basic information for irradiated food detection using a new high performance liquid chromatography (HPLC) analytical procedure. The procedure was established using an automated pre-column derivatization with 4-fluoro-7-nitro 2,1,3-benzoxadiazole (NBD-F) followed by reverse phase HPLC and LASER fluorometric detection. The limit of detection (LOD) was 0.06 ng on-column and the linear range for calibration was 0.06 to 50 ng for the tyrosine derivatives. The relative standard deviation was 10% to 12%. The amounts of the tyrosine isomers increased with levels of irradiation. Irradiation at low temperature with reduced oxygen decreased the isomer yields. In the pH range of 5 to 7, the amount of product was not changed significantly by pH, outside this range, the pH did have an effect on product generation. At constant dose levels the yields of tyrosine isomers initially increased with phenylalanine concentration, although, with further increases in phenylalanine a reduction in the absolute amounts was observed. Dose rates varying from 0.5 kGy/h to 10 kGy/h had no significant effect on tyrosine isomer formation if a total of 10 kGy was used in each case. In addition, demonstrating the usefulness of this new analytical technique for *o*-tyrosine determination, these studies suggest that the presence of *o*-tyrosine is another parameter indicative of gamma irradiation.

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

INTRODUCTION

Our program to develop analytical methods for the detection of food contaminants, such as pesticides and mycotoxins[1] has been expanded to include detection of radiolytic products. Gamma irradiation of food is used to reduce microbial population, inhibit sprouting, and destroy parasites. Foods so treated must be labeled.[2–4] However, to date, few methods for detecting irradiated food have been established and approved as authorized procedures.[5] The use of *o*-tyrosine methods has been investigated for years.[6] Establishing a chemical method for detecting irradiated foods is made difficult by the low-level analytes in complex matrix, and poor dose response of radiolytic products.

When phenylalanine solution is irradiated with gamma-rays, the phenylalanine is oxidized to give tyrosines (*ortho-*, *meta*, and *para*-tyrosine) by hydroxyl radicals that are generated by ionization of water, as shown in Fig. 1. This phenomenon was recognized in the 1920's.[7] Since then

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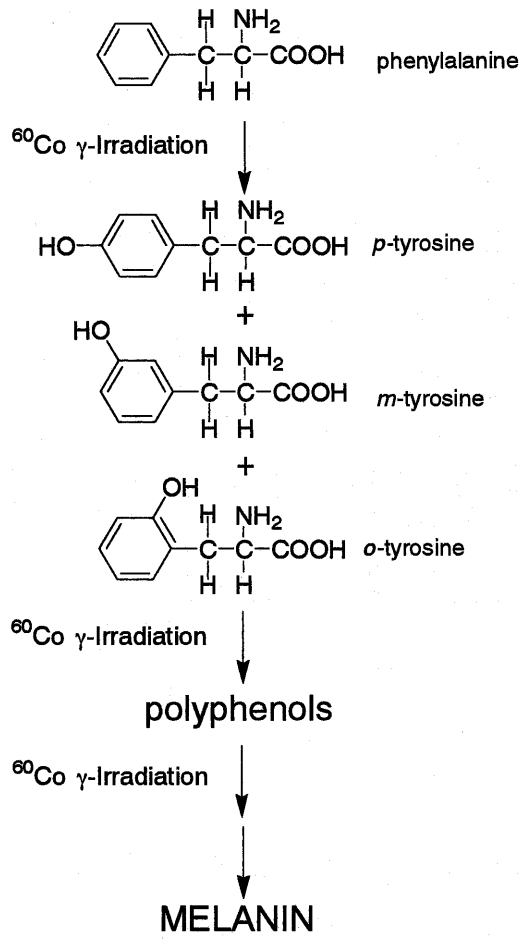

Fig. 1. Schematic Chart of Radiolytic Reaction of Tyrosine and Phenylalanine in Aqueous Solution by Gamma-Irradiation of ^{60}Co

there have been extensive studies on oxidation of phenylalanine to give tyrosines have been performed.[8-11] Radiolytic products have been oxidized to give a mixture of polyphenols and melamine.[12] The basic phenomena in irradiated solution are known,[13] but details of product formation and irradiated food detection have yet to be elucidated. Radiolytic products that are obtained at high-dose (20—100 kGy) were extensively studied by Simic[9] and by the U.S. army in the 1950's.[7] They were based on the preliminary findings of amino acid oxidation made by Stenstrom and Lohmann[14] in the 1920's and others. Analytical techniques and related skills have obviously improved since then.

The *o*-tyrosine method has good potential for detection of irradiated food because many foods contain a constant level of precursors (phenylalanine residues) for radiolytic products (tyrosine isomers) in protein samples and irradiated foods can be detected by the same standard. The ESR detection method for boned meat, the thermoluminescence method for spices, and the half-embryo method for citrus require born, inorganic dusts, and seeds in sample, respectively.[5] Such methods strongly depend on specific materials. The *o*-tyrosine method does not.

However, *o*-tyrosine is not "an unique radiolytic product" and some amounts (0.03—0.08 ppm) are detected even in non-irradiated foods.[6),15] Studies have reported *o*-tyrosine formed *via* enzymatic oxidation in tissue[16] and *via* solvent used for sample preparation.[8] Therefore, it is difficult to estimate the absorption dose based on the *o*-tyrosine level in a sample, and to determine whether that sample has been irradiated or not, without background information.[17] The background levels of *o*-tyrosine in foods differ from sample to sample. As a result, a numerical detection limit for irradiated foods can't be set based on the *o*-tyrosine level in a sample.

A second problem is poor reproducibility.[15] Some have detected irradiated foods using this method, while others have not.[18] The traditional method for the detection includes fluorometric detection with HPLC separation monitoring at 305-nm emission (excitation at 275 nm) with a detection limit of about 1 ng. Another method using gas chromatography (GC) with mass spectrometry or flame ionization detector requires derivatization of analyates by bis-(trimethylsilyl)-trifluoroacetamide[8,10,19] and has a detection limit over 10 ng. These early GC procedures provide for either non-specific detection with relatively high sensitivity (GC-FID) or specific detection with low sensitivity (GC-Mass).

The assessment of low levels of *o*-tyrosine in food or complex matrix required new sensitive specific analytical procedures. Here we report development of a sensitive assay system using derivatization of sample by fluorometric dye of NBD-F[20] with LASER fluorescence detector. This system enables one to analyze amino acids more sensitively than ever before (a capability to detect less than 10 pico grams of 4-fluoro-7-nitro 2,1,3-benzoxadiazole (NBD-F) derivative). This detector is designed only for detection of NDB-derivatives and equipped with a small cell for peak separation. To eliminate lengthy derivatization procedures, automated pre-column derivat-

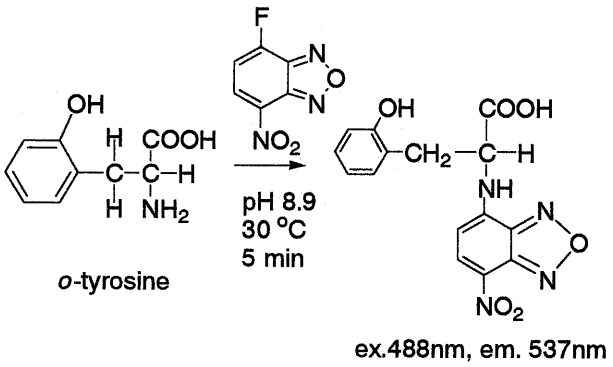

Fig. 2. Schematic Chart for *o*-Tyrosine Derivatization by NBD-F for Fluorometry

ization was adopted, as shown in Fig. 2. The system ensures reproducibility of pipetting and an accurate time course for the derivatization. The combination of the automatic fluorometric derivatization system and high performance HPLC column enable one to analyze the radiolytic products specifically and precisely. This paper also describes effects of gamma-irradiation conditions (temperature, oxygen level, pH *etc.*) on *o*-tyrosine formation to assess the scope and limitation of the method.

MATERIALS AND METHODS

Irradiation Apparatus —— 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.

HPLC System —— A block diagram of the analytical system used to obtain control data for each device and data from the detection system are shown in Fig. 3. A schematic diagram of the pre-column HPLC system used for *o*-tyrosine derivatization is shown in Fig. 4. The system consisted of a Shimadzu model LC10A pump HPLC with a LASER fluorescence detector (Tosoh mode LA 8010 monitor, operated at 488 nm for excitation and 550 nm for emission), an automated sampler (Shimadzu model SIL10A), a degasser (Shimadzu model DGU10A), and a column oven (Shimadzu model CTO10A). An ODS column (250 mm × 4.7 mm i.d., 5mm; Shimadzu ODS-2 (Shimadzu, Co.)) was used. The column oven was maintained at 45℃. Injections were performed by an autosampler with a completely filled 20 μl injection loop. Data were processed with a Shimadzu model CR7A data processor.

Derivatization —— Derivatization consisted of mixing 20 μl samples with 10μl of saturated borate, and 10 μl of NBD-F acetonitrile solution, holding for 10 min, adding 0.1 N hydrochloric acid, and mixing twice. This derivatization procedure was performed automatically in the autosampler, as shown in Fig. 4.

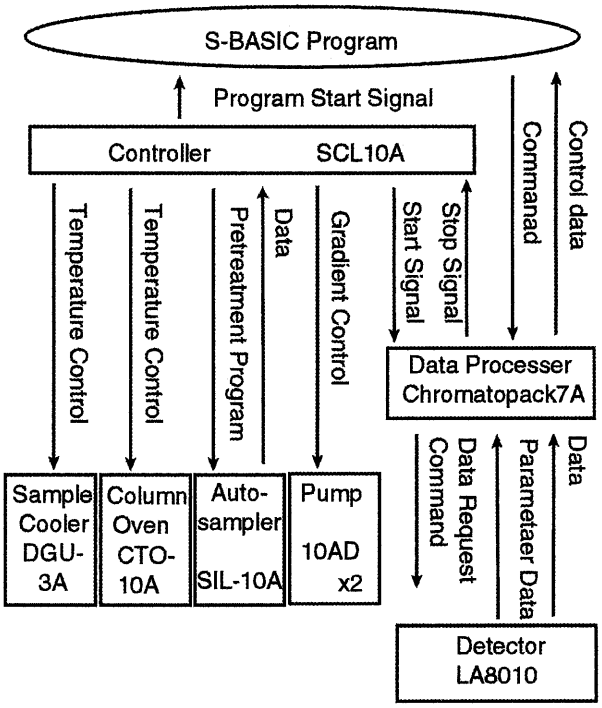

Fig. 3. The Block Diagram of Control Data and Data Signals for the Pre-column Derivatization System

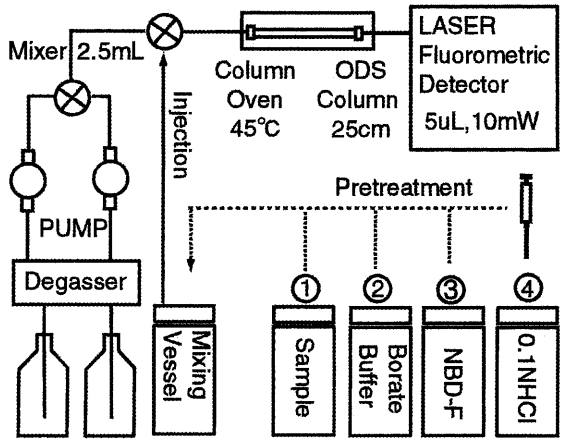

Fig. 4. The Schematic Diagram of System Configurations for the HPLC System

Reagents and Other Materials —— Amino acid standards included phenylalanine > approx. 98%, *o*-tyrosine, *meta*-tyrosine, and *para*-tyrosine > approx. 95%. The standards were purchased from Sigma, St. Louis, MO.

All reagents for analysis were of Japanese Industrial Standards (JIS) extra pure grade. These are

compatible with ACS grade. Water was of HPLC grade (Ciba Merck, Co.).

Reagents for Derivatization —— Reagents for derivatization included NBD-F solution, buffer solution, and stop solution. Reagent solution for derivatization was prepared by dissolving 10 mg of NBD-F in 10 mL acetonitrile. Saturated borate solution was used as the derivatization buffer. The stop solution was 0.1 N hydrochloric acid.

Mobile Phase for HPLC —— Solvent A was 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 B solution was maintained at 30% for 20 min, then rose 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 —— One hundred milligrams of each amino acid was dissolved in 100 mL of water.

Irradiation Procedure —— Five milliliters of aqueous phenylalanine solution was placed in a 10 ml Pyrex tube with a stopper. The sample was irradiated at 6 kGy/h with the plate-type source for precision irradiation. Routinely, a sample was irradiated with the rod-type source at an appropriate dose rate. Irradiation was conducted at room temperature, in a vessel with a stopper. After irradiation, the samples were stored at −20℃.

Dosimetry —— Absorbed doses were measured with CPI (Chemical Process Indicators, NAS, OH) and Radix RN-15 (Radie Kogyou, Japan) was used for calibration.

Caution —— The gamma-irradiator should be operated with careful monitoring and supervision by someone experienced in irradiation. NBD-F and some organic solvents used in this study are suspected carcinogens. Handle them with care.

RESULTS AND DISCUSSION

Pre-column Derivatization and Analytical Conditions

To establish pre-column reaction conditions for automated pipetting, several reaction conditions were examined by introducing standard tyrosine solution. The pretreatment procedure for derivatization was programmed into the auto-sampler, and included pipetting 10 μl of reaction buffer, 10 μl of sample, and 10 μl of NBD-F solution, mixing the solution, standing at an appropriate temperature until the derivatization was complete, pipetting 400 μl of stop solution (0.1 N HCl), and injecting 20 μl of the reaction mixture for HPLC. Pipetting time and tube-washing time had to be considered, as they affect detection limits. The time course of the reaction was examined to obtain the optimal reaction time from 0 to 180 min. The fluorescence intensity of the reaction mixture at 550nm topped 15 min after the reaction started. The reaction temperature and pH were also examined, by changing from 30 to 70℃ at constant pH and reaction time and pH 6.5 to 10 at constant temperature and reaction time. The intensity of the reaction mixture increased as the temperature rose. Due to instrument limitation and reproducibility, 45℃ was chosen. Fluorescence was most intense at pH 8. Pipetting less than 5 μl did not give accurate results with our system. For 15 min, the fluorescence of the reaction mixture showed a steady intensity and then gradually decreased. Program parameters for the auto-sampler were established from the results.

Chromatographic conditions were examined to separate target peaks from the fluorescent interference which came from pre-column derivatization treatment, by changing the gradient rate of mobile phases. As shown in Fig. 5a,b (a for standard tyrosines and (b for 10 kGy irradiated phenylalanine sample, the target peaks were successfully separated from each other. The gradient of the mobile phase was very critical to the separation. A good technique should be applied to preparing the mobile phase solutions and a precision pump system is recommended. Dead volumes in the piping system and the injection system must be minimized, otherwise peak width will broaden. The LOD was 0.06ng and the linear

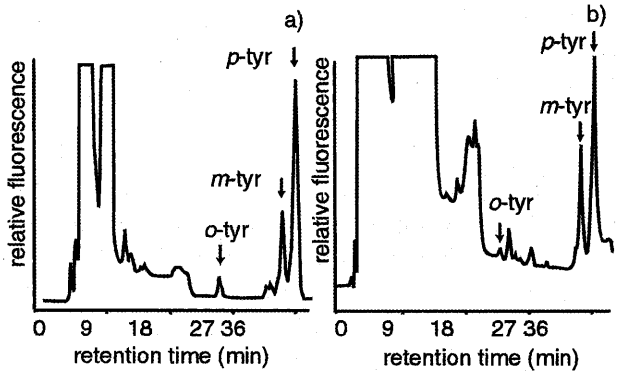

Fig. 5. Chromatograms for (a) Standard Tyrosines (3.5 ng each) and (b) Irradiated Phenylalanine Solution (1 mg/ml) at 10 kGy

range of the calibration curves for the tyrosine derivatives was 0.06 to 50 ng. The relative standard deviation was between 10% and 12%.

Influence of Irradiation Conditions

Effect of Temperature

Effects of temperature on the formation of tyrosine were examined. The samples were irradiated with 10 kGy at −40, 0, 15℃ and the results are shown in Table 1.

At −40℃, the solution was frozen and did not give any isomer of tyrosine. At 0 and 15℃, 30—60 μg/ml and 40—80 μg/ml of tyrosine formed, respectively. Results were mostly consistent with those by Krajnik *et al.*,[21] who observed no difference between the yield at 0℃ and 20℃. But our results show the formation of tyrosines was dependent on the irradiation temperature at all ranges we examined. The most effective temperature for sterilization by the gamma irradiation is around 0℃, at which sufficient *o*-tyrosine is generated to be detected. As to the *o*-tyrosine formation, the difference between 0℃ and −40℃ was about 10%, and some temperature dependence of amino acid oxidation was observed.

Table 1. Effects of Temperature on Formation (μg/ml) of Tyrosines at 10 kGy[1]

Temperature (℃)	-40	0	15
o-Tyrosine	0	0.034	0.04
m-Tyrosine	0	0.038	0.047
p-Tyrosine	0	0.06	0.078

1) Irradiation conditions: dose rate 2.5 kGy/h; pH, neutral; phenylalanine level, 1 mg/ml.

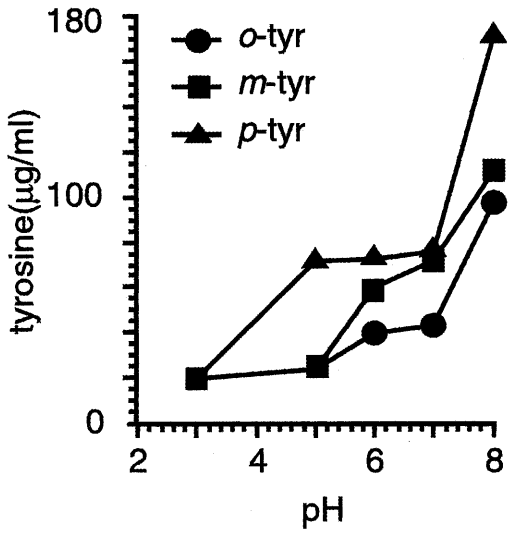

Fig. 6. Effects of pH on Formation of Tyrosines at 10 kGy

Irradiation conditions: temperature, ambient; dose rate 2.5 kGy/h; phenylalanine level, 1 mg/ml.

Effect of pH

The pH of the phenylalanine solution affected the formation of tyrosine. As shown in Fig. 6, 20 μg/ml, 20—72 μg/ml, and 100—165 μg/ml of tyrosines formed at pH 3, pH 6—7, and pH 8, respectively. Regarding *o*-tyrosine, a 2—5-fold increase was observed with increase of the pH of the solution. Thus, pH of sample is very important for the formation of *o*-tyrosine and detection limit.[11] The effects of pH on oxidation of glycine have been reported and the pH dependence of yield is due to the zwitter ion character.[9] However, the results can also be rationalized by the fact that irradiated water is reductive under acidic conditions and oxidative under alkaline conditions,[13] and the initial products (aqueous electrons, hydrogen radicals, hydroxyl radicals *etc.*) in irradiated water are also affected by pH.[22] For example, under alkaline conditions hydrogen radicals readily react with oxygen in solution to give hydroxyl radicals.

The pH of an individual practical sample is hard to control, therefore this procedure has limitations and requires precausional treatments to prevent changing pH of the sample. For example, meat and seafood should be protected from attack by microbial-organisms and kept at a neutral pH. Fermented foods and foods pH-adjusted to control microorganisms or for other reasons may be difficult to determine by this procedure.

Effect of Phenylalanine Concentration

The effects of phenylalanine concentration on yields of tyrosines were examined. Aqueous phenylalanine solutions (0.25—4 mg/ml) were irradiated at room temperature for 10 kGy. The phenylalanine solution of 2 mg/ml gave the maximum tyrosine concentration (78 μg/ml, 43 μg/ml, and 42 μg/ml of *para*, *meta*, and *ortho* isomers, respectively) as shown in Fig. 7. At this concentration (2 mg/ml), radicals formed by irradiation were reacted efficiently. This illustrates that the hydroxylation by gamma irradiation is affected by components which exist around the phenylalanine moieties. Therefore, the reproducibility of the *o*-tyrosine method for irradiated food identification with actual complex matrix may be poor. Degradation of

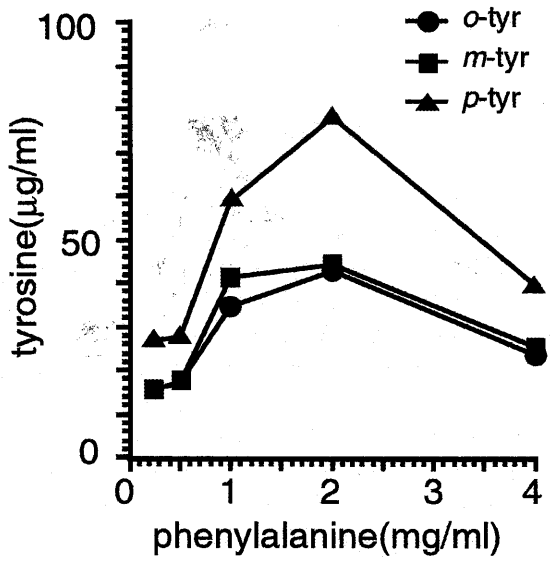

Fig. 7. Effects of Phenylalanine Levels in Aqueous Solution on Formation of Tyrosines at 10 kGy

Irradiation conditions: temperature, ambient; dose rate 2.5 kGy/h; pH, neutral.

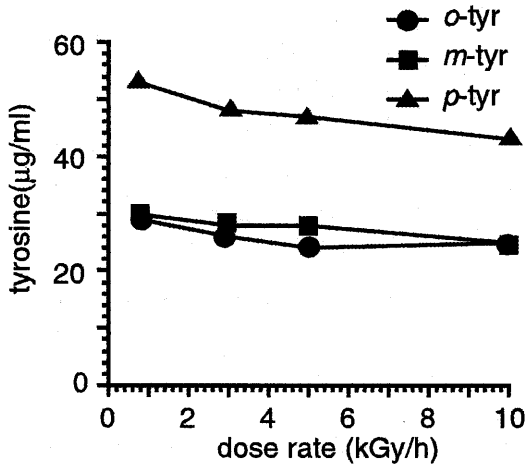

Fig. 8. Dose Rate (0.75—10kGy/h) Effects at 10 kGy-Dose

Irradiation conditions: temperature, ambient; pH, neutral; phenylalanine level, 1 mg/ml.

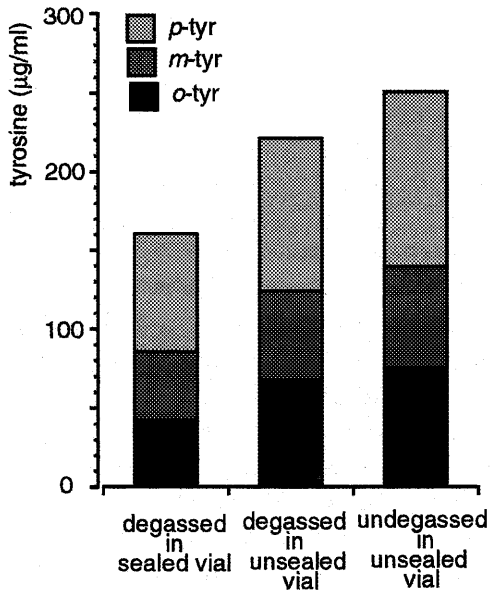

Fig. 9. Effects of Low Oxygen Level on the Formation of Tyrosines at 10 kGy

Irradiation conditions: temperature, ambient; dose rate 2.5 kGy/h; pH, neutral; phenylalanine level, 1 mg/ml. Tyrosines obtained by irradiating phenylalanine solutions in a) degassed and sealed vessel, b) degassed and unsealed vessel, and c) non-treated and unsealed vessel.

phenylalanine by high-voltage cathode rays has been reported and the degree of decomposition decreases with increasing concentration of amino acids.[23]

Effect of Dose Rate

The effects of dose rate were examined over the range from 0.75 kGy/h to 10 kGy/h at room temperature. The aqueous phenylalanine solutions gave 107, 60, and 59 μg/ml (total 112 μg/ml) of *p*, *m*, and *o*-tyrosines, respectively at 0.75 kGy/h, and, 43, 28, and 25 μg/ml (total 96 μg/ml) of *p*, *m*, and *o*-tyrosines, respectively, at 10 kGy/h, as shown in Fig. 8. There was no significant difference between these results.

Effect of Low Oxygen Level

The effects of oxygen level in samples were examined using degassed sample solutions in vials, degassed sample vials opened to air immediately before irradiation, and non- treated sample. The results are shown in Fig. 9. Total tyrosine concentration ranged from 160 μg/ml in degassed samples to 230 μg/ml in unsealed vials. A 143% increase was observed. Low oxygen pressure affects tyrosine formation. This means oxygen in the air catalyzes the oxidation of phenylalanine.[10] Krajnik *et al.*[21] reported a similar observation. Active particles formed by the aqueous electron reaction play a role together with superoxide anion radicals in the oxidation.[11,13,22]

Dose Response

Dose response was observed in the range from 0.75 to 10 kGy as shown in Fig. 10. Samples in water were irradiated at room temperature at 2.6 kGy/h. This illustrates that the tyrosines are stable enough for a dose response. However, they degraded further at high doses, and higher dose reduced the G-value (Table 2). The G-values are

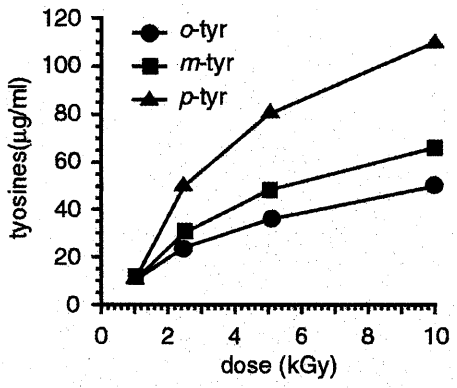

Fig. 10. Dose Response (0—10 kGy)

Irradiation conditions: temperature, ambient; dose rate 2.5 kGy/h; pH, neutral; phenylalanine level, 1 mg/ml.

Table 2. Dose Dependency of G-Value

Dose (kGy)	*o*-Tyr	*m*-Tyr	*p*-Tyr
1	0.53	0.53	0.53
2.5	0.51	0.64	1.07
5	0.38	0.51	0.85
10	0.27	0.35	0.59

comparable with earlier results.[10]

In accordance with early observations.[24] the color of the irradiated phenylalanine solution at higher dose was deeper than that at lower dose under our experimental conditions. As shown in Fig. 1, the tyrosines that formed at high dose with sufficient oxygen in the solution reacted readily with the irradiation-induced oxidants to give secondary products (for example, polyphenols), therefore, the observable G-values of tyrosines were reduced at higher dose.[12] The polyphenols are further reacted with to give melanin, which shows a color characteristic of the solutions of phenylalanine or tyrosine.[24]

In conclusion, to determine tyrosine concentration, a new analytical procedure has been developed. The method is more specific to tyrosine and can determine low levels of tyrosine in sample. The radiolytic reaction of phenylalanine in water was affected by several irradiation conditions: pH, temperature, oxygen level and dose. Those factors may affect the results of irradiated food identification. Further study using irradiated food is under way.

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