An HPLC Method To Determine *o*-Tyrosine in Chicken Meat

Noemi Chuaqui-Offermanns* and Tom McDougall

Radiation Applications Research Branch, AECL Research, Whiteshell Laboratories, Pinawa, Manitoba, Canada R0E 1L0

The lack of analytical methods to establish whether or not a food product has been irradiated, and to what dose, if a major obstacle to the wider use of the food irradiation process. *o*-Tyrosine produced during irradiation of protein-containing foods appears to be a promising marker for this purpose. An HPLC/fluorescence method that allows accurate quantitation of 0.1 ng of *o*-tyrosine has been developed. The method involves freeze-drying of the sample, acid hydrolysis, solid-phase extraction, fractionation by HPLC, and, in some cases, a second chromatographic separation (HPLC) of the collected fraction. This method was used to determine the radiation dose yield of *o*-tyrosine in irradiated chicken breast. The results show that there is a linear relationship between the irradiation dose and the yield of *o*-tyrosine in irradiated chicken meat.

INTRODUCTION

In principle, any radiation-induced chemical change occurring in a foodstuff can be used as a marker to detect and quantify radiation treatment. Since these changes are very small at the approved doses for radiation processing of foods (≤10 kGy), very sensitive analytical methods are required to detect them.

In recent years, the interest in using ionizing radiation as a means of raw meat preservation has been increasing worldwide. The major component of raw meats is water, which when exposed to ionizing radiation decomposes, forming free radicals:

$$H_2O \xrightarrow{\gamma} HO^{\bullet} + e^-_{aq} + H^+$$

The main target for the free-radical species is the major nonaqueous component of raw meats, namely, proteins (Grootveld and Jain, 1989; Simic et al., 1989). Hydroxylation of amino acids occurs readily, with rate constants of the order of 10^9 $mol^{-1} \cdot s^{-1}$. Phenylalanine, an amino acid found in most food proteins, reacts with hydroxyl radicals, giving rise to three isomeric products: *o*-, *m*-, and *p*-tyrosine (Dizdaroglu and Simic, 1981).

Several papers have claimed the suitability of *o*-tyrosine as a marker to detect and quantify radiation treatment in protein-containing foods (Karam and Simic, 1988, 1989; Simic et al., 1988; Grootveld and Jain, 1989); others, however, have disputed that claim (Hart et al., 1988; Meir et al., 1988; Halliwell et al., 1988; Willemot et al., 1989). This controversy indicated to us that a validation study was required.

To conduct this validation study, we needed a method to accurately quantify *o*-tyrosine in protein-containing foods. A review of the existing methods showed either that they were too cumbersome and required highly specialized equipment or that their resolution was inadequate. Therefore, the development of a simple and accurate analytical method to quantify *o*-tyrosine in meat became the first objective of our project.

MATERIALS AND METHODS

Apparatus. The chromatographic system consisted of (i) a reversed-phase 4.6 × 250 mm 5-μm Selectosil (Phenomenex) C_{18} column with a Novapak C_{18} precolumn cartridge (Waters), (ii) an Isco Model 2300 pump connected to two solvent ports via a three-way stream electrically operated switching valve, (iii) a Shimadzu Model RF-535 fluorometric detector, and (iv) a Shimadzu Model C-R3A data processor.

Sample Preparation. Irradiated and nonirradiated chicken parts were skinned and boned, and the fat adhering to the meat was carefully scraped away with a knife. The clean poultry part (leg or breast) was freeze-dried for approximately 72 h until constant weight. The dried samples were homogenized in a blender, placed in tightly sealed plastic containers, and stored in a desiccator over a desiccant.

Hydrolysis. Five milliliters of 6 N HCl was added to 250 mg of sample in a Pierce protein hydrolysis tube. The tube was cooled to −20 °C, evacuated at room temperature until its content was completely thawed, sealed, and then heated for 1 h at 150 °C. The evacuation procedure was repeated twice to ensure the total removal of oxygen.

Cleanup. Hydrolyzed samples were filtered through a 0.45-μm Millipore filter; 1.0 mL of filtrate was cleaned up by a solid-phase extraction, which uses a C_{18} reverse-phase Sep-Pak cartridge. The cartridge was conditioned by prewetting it with 10 mL of methanol and then flushing with 10 mL of water. The loaded sample was eluted with 4 mL of a 95% water and 5% acetonitrile mixture. The eluted fraction containing the sample was evaporated to dryness and dissolved in 500 μL of 2 N HCl.

HPLC. Twenty-microliter fractions were injected into a reverse-phase Selectosil C_{18} 5-μm analytical column, 4.6 mm i.d. × 250 mm. Prior to injection, the column was equilibrated either by washing it for 35 min with eluant A (1% acetonitrile, 1.5% dihydrogen phosphate, and water, pH 4) at a flow rate of 1 mL/min or by using a binary-solvent system. The binary-solvent system consisted of the eluant A described above and eluant B (50% acetonitrile, 0.75% sodium dihydrogen phosphate, and water). The column was first equilibrated for 35 min with eluant A and then washed for 5 min with eluant B at a flow rate of 1.2 mL/min and again for 20 min with eluant A. In both cases the sample was eluted with eluant A at a flow rate of 1.2 mL/min for 20 min.

In the case of the single-solvent system, the column was washed after each injection with eluant A for 45 min. For the binary-solvent system, the column was reequilibrated after each injection by washing it with eluant B for 5 min and with eluant A for 20 min. The selection of either solvent system depends on the sample characteristics. Some samples contain interfering compounds that are not eluted by eluant A alone, resulting in a poorer performance of the column and, consequently, inadequate separation of *o*-tyrosine.

o-Tyrosine was detected fluorometrically (λ excitation = 275 nm, λ emission = 305 nm). The retention time of *o*-tyrosine was determined by using standard solutions of the pure analyte and by spiking hydrolyzed samples with known amounts of *o*-tyrosine standard.

In some samples, the amount of fluorescent impurities, mainly

0021-8561/91/1439-0300$02.50/0

p-tyrosine and phenylalanine, prevented the base-line separation of o-tyrosine. This resulted in an integration of the peak of interest on the tail of eluting impurities, leading to inaccurate quantitations. The problem was easily solved by collecting the fraction containing the o-tyrosine, eluting at a previously determined retention time, and then conducting a second chromatographic separation. The collected fraction was evaporated to dryness and dissolved in 200 μL of water. The second chromatographic separation fo the fraction uses a single-solvent system consisting of 1% acetonitrile, 1% sodium chloride, and water; under these conditions a base-line separation of o-tyrosine was obtained.

The column was washed at the end of each working day with a 80% acetonitrile and 20% water solution for 45 min.

RESULTS AND DISCUSSION

Selection of Columns. Several reverse-phase columns from different suppliers were tested for their ability to separate o-tyrosine in chicken samples. Two columns were selected. The first one was a 3-μm reverse-phase Nova-Pak C_{18} Radial-Pak chromatography cartridge, 8 mm i.d. × 10 cm.

The main problem encountered with this column was its short service life, with poor resolution and development of high back-pressure after a relatively small number of injections (approximately 60) being the main manifestations of impending column failure.

The second column selected was a 5-μm, C_{18} reverse-phase Selectosil Phenomenex stainless steel column, 4.6 mm i.d. × 250 mm. The service life of this column was longer (approximately 300 injections) than that of the Radial-Pak column. This service life was increased further, to approximately 400 injections, by introducing the cleanup procedure described earlier. The other advantage common to all stainless steel columns, as opposed to cartridges, is that they can be opened, allowing solution of problems that cause overpressure.

Selection of Mobile Phase. The first mobile phase (MP1) used was the one reported by Meir et al. (1989). It comprised three eluants: eluant 1, 1% acetonitrile, 1% sodium chloride, water; eluant 2, 50% acetonitrile, 0.5% sodium chloride, water; and eluant 3, 80% acetonitrile, water.

Because the samples to be analyzed are strongly acidic (2 N HCl), and the solvent system used lacked buffering capacity, several metal components of the apparatus corroded. Two approaches were taken to avoid this problem. The first involved buffering of the sample after the cleanup step, to a pH between 3 and 5. It was found that samples neutralized to pH above 1 were unstable.

The second approach taken was to use a buffered eluant (MP2) consisting of 1% acetonitrile, 1.5% sodium dihydrogen phosphate, and water. Figure 1 illustrates a typical separation obtained with this eluant. The o-tyrosine peak is well separated and can be easily integrated without the need to collect a fraction for reinjection. However, as illustrated in Figure 2, this type of separation was not always obtained. Consequently, to determine accurately the amount of o-tyrosine, a fraction eluting at the previously determined retention time was collected and separated by HPLC again as described earlier.

It should be emphasized that all the development work was done with unirradiated samples. The levels of o-tyrosine in these samples are much lower than those expected in irradiated samples. In other words, the method is being tested under conditions requiring extreme sensitivity.

Radiation Tests. Two whole fresh chicken breasts were placed on ice and irradiated to 0, 1, 2, 3, and 4 kGy in a ^{60}Co Gamma Cell 220 (dose rate = 0.17 kGy/min). Each

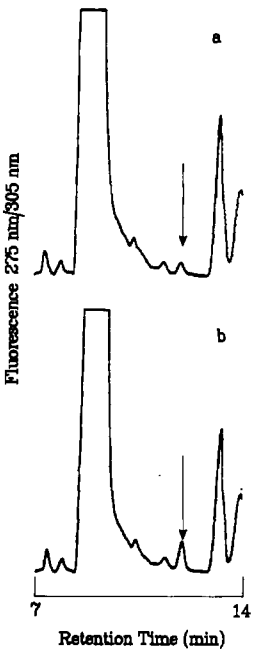

Figure 1. Separation of o-tyrosine from a hydrolysate of chicken breast after cleanup (2 N HCl). Column: 5-μm C_{18} reverse-phase Selectosil Phenomenex stainless steel column. Mobile phase: acetonitrile 1%, 0.1 M NaH_2PO_4 buffer, pH 4, 1.5%, water. (a) Sample, recorder attenuation = 1; (b) spiked sample, recorder attenuation = 1. Arrows indicate the position of the o-tyrosine peak.

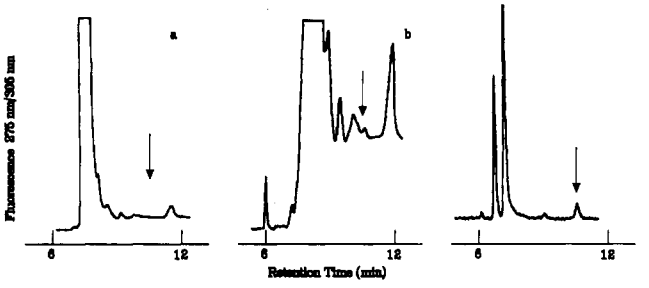

Figure 2. Separation of o-tyrosine from hydrolysate of chicken breast after cleanup (2 N HCl). Column: 5-μm C_{18} reverse-phase Selectosil Phenomenex stainless steel column. Mobile phase: acetonitrile 1%, 0.1 M NaH_2PO_4 buffer, pH 4, 1.5%, water. (a) Sample, recorder attenuation = 4; (b) sample, recorder attenuation = 1; (c) fraction collected from the run shown in (b). Arrows indicate the position of the o-tyrosine peak.

irradiated breast was analyzed for its o-tyrosine content as described earlier (four samples per dose). As shown in Figure 3, the yield of o-tyrosine is proportional to the dose. This linear dependence is described by the following equations:

$$\text{o-tyrosine (ppm/wet weight basis)} = 0.120 \times \text{dose (kGy)} + 0.303 \quad \text{(chicken 1)}$$

$$\text{o-tyrosine (ppm/wet weight basis)} = 0.127 \times \text{dose (kGy)} + 0.120 \quad \text{(chicken 2)}$$

The intercepts of 0.303 and 0.120 represent the content of o-tyrosine in unirradiated chicken samples. The background levels of o-tyrosine determined in samples from unirradiated portions of chickens 1 and 2 were 0.3 ± 0.05 and 0.120 ± 0.012 ppm per wet weight (70% moisture content), respectively. These results are in very good

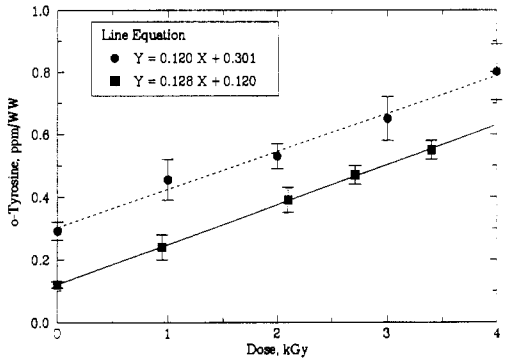

Figure 3. Radiation yield of *o*-tyrosine in chicken breast irradiated at 0, 2, 3, and 4 kGy. Each plotted value represents the mean content ± the standard deviation ($n = 4$) of *o*-tyrosine in parts per million (wet weight, 70% moisture). (●) Concentration of *o*-tyrosine chicken 1; (■) concentration *o*-tyrosine chicken 2.

agreement with the extrapolated values, and they also indicate that the background levels of *o*-tyrosine in unirradiated chicken vary from sample to sample.

CONCLUSIONS

From this study we conclude that the 5-μm C_{18} reverse-phase Selectosil Phenomenex is superior to the Nova-Pak C_{18} Radial-Pak to quantify *o*-tyrosine in poultry samples. Mobile phase MP2 is more effective than mobile phase MP1 in separating *o*-tyrosine from impurities. The cleanup procedure is essential to the method. The method developed is simpler and more accurate than those previously reported. It allows the base-line separation of *o*-tyrosine from impurities present in relatively high concentrations. For example, the ratio of main impurities to *o*-tyrosine is 10^5:1 in unirradiated samples. However, since samples from one chicken are chromatographically different from samples taken from another chicken, it is almost impossible to have a very rigid HPLC procedure that could be applied to every situation. The method described here is flexible. Alternatives were given for cases in which the resolution is affected either by the size of the interferences (analyte eluting on the tail of a large peak) or by the analyte not being fully separated from a coeluting impurity. The yield of *o*-tyrosine in irradiated chicken breast is proportional to the dose. Since variable levels of *o*-tyrosine were found in unirradiated chicken breast (0.302 ± 0.05 and 0.12 ± 0.012 ppm wet weight), background levels must be determined in samples of different origin, as well as in samples stored under different conditions. This work is already in progress.

LITERATURE CITED

Dizdaroglu, M.; Simic, M. G. Hydroxycyclohexadienyl peroxy radicals. In *Oxygen Radicals*; Rodgers, M. A. J., Powers, E. L., Eds.; Academic Press: New York, 1981; p 619.

Grootveld M.; Jain, R. Methods for the detection of irradiated foodstuffs: Aromatic hydroxylation and degradation of polyunsaturated fatty acids. *Radiat. Phys. Chem.* **1989**, *34* (6), 925.

Halliwell, B.; Grootveld, M.; Gutteridge, M. C. Methods for the measurement of hydroxyl radicals in biochemical systems: deoxyribose degradation and aromatic hydroxylation. In *Methods of Biochemical Analysis*; Glick, D., Ed.; Wiley: New York, 1988; Chapter 2, p 59.

Hart, R. J.; White, J. A.; Reid, W. J. Technical Note: Occurrence of o-tyrosine in non-irradiated foods. *Int. J. Food Sci. Technol.* **1988**, *23*, 643.

Karam, L. R.; Simic, M. G. Detecting irradiated foods: use of hydroxyl radical biomarkers. *Anal. Chem.* **1988**, *60*, 1117A.

Karam, L. R.; Simic, M. G. Mechanisms of free radical chemistry and biochemistry of benzene. *Environ. Health Perspect.* **1989**, *82*, 37.

Meier, W.; Bürgin, R.; Fröhlich, D. Analysis of o-tyrosine as a method for the identification of irradiated chicken meat. *Beta-Gamma* **1988**, *1*, 34.

Simic, M. G.; Bergtold, D. S.; Karam, L. R. Generation of oxy radicals in biosystems. *Mutat. Res.* **1989**, *214*, 3.

Willemot, C.; Chayer, D.; Gosselin, C.; Couture, R.; Drouin, S.; Castaigne, F. *Can. Inst. Food Sci. Technol. J.* **1989**, *22* (5), 350.

Received for review June 18, 1990. Revised manuscript received August 30, 1990. Accepted September 21, 1990.

Registry No. *o*-Tyrosine, 709-16-0.