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# Analysis of Carbon Monoxide in Commercially Treated Tuna (*Thunnus* spp.) and Mahi-Mahi (*Coryphaena hippurus*) by Gas Chromatography/Mass Spectrometry

COLLIN R. ANDERSON AND WEN-HSIN WU*

U.S. Food and Drug Administration, Seafood Products Research Center/Pacific Regional Laboratory Northwest, 22201 23rd Drive SE, Bothell, Washington 98021

A simple and confirmative method for quantitative determination of carbon monoxide in tuna and mahi-mahi tissues using GC/MS, following chemical liberation of CO into headspace, is described. Carbon monoxide in recent years has been employed by the fishery industry to preserve fresh appearance in selected species of finfish during frozen storage, particularly in vacuum-packaged products. Indigenous CO contents of fresh Ahi tuna and mahi-mahi were examined using the method described in this study and found to be close to or less than 150 and 100 ng/g, respectively. Commercially CO-treated, vacuum-packaged tuna from multiple sources consistently showed CO level near or greater than 1 μg/g, while CO level in the only CO-treated frozen mahi-mahi sample was in the 500 ng/g range. The difference between untreated and treated specimens was in the range of 1 order of magnitude and thus suggested an easy quantitative and confirmative method of CO using widely available instrumentation that may be potentially useful for regulatory purpose in determining whether a commercially available product has been exposed to CO even if not labeled as such.

**KEYWORDS: Carbon monoxide; GC/MS; headspace; tuna; mahi-mahi**

## INTRODUCTION

To increase sales and add values, the seafood industry is continuously attempting to improve quality and create attractive attributes in aquatic foods for consumers. One such action in recent years has led to the use of carbon monoxide (CO) or tasteless smoke (TS), which contains CO gas to fix the bright red color in the dark muscle tissue of fish such as tuna. Under natural condition, as the result of oxidation tuna muscles are highly susceptible to discoloration after being cut and particularly during the subsequent freezing storage, which leads to a brown color often termed "chocolate" tuna and therefore presents a great challenge to commercial fisheries. Besides color preservation, the intended use for gas treatment has also been reported to preserve other qualities such as "...taste, aroma, texture" (*1*). A number of studies have demonstrated the positive effects, mainly in the improvement of color stability, of low amounts of CO in modified atmosphere packaging (MAP) and its purported safety for packaged beef and pork products (*2*−*7*). Research conducted at the University of Florida has shown many beneficial effects toward fishery products, such as mahi-mahi and Yellowfin tuna, treated with CO and filtered smoke (FS) (*8*). Treated tuna had significantly enhanced red color (a-values), reduced lipid oxidation, and lowered aerobic bacterial counts leading to extended shelf life. Histamine production, however, continued for both CO- and FS-treated products, albeit at a slower rate as the CO treatment was increased. More recently, research at the Danish Institute for Fisheries Research demonstrated significant histamine formation by marine psychrotolerant bacteria in vacuum-packaged tuna during refrigeration storage (*9*). These findings raise substantial concerns over the potential for product abuse leading to high levels of histamine in the product, which may be disguised by an otherwise "fresh-looking" piece of fish. In addition to tuna, apparently fillets of tilapia (*Oreochromis* spp.) produced in Asian countries for export are often treated with CO to prevent brown color development before packaging for frozen storage (personal communication, *10*), which may potentially be a safety and labeling concern.

The color of the dark muscle tissue in fish is the result of the oxidation state of myoglobin protein and, perhaps to a lesser extent, hemoglobin. Both serve to bind oxygen ($O_2$) for cellular use and contain a heme group with an Fe(II) center bound to four nitrogen molecules of the porphyrin ring. The fifth bond orbital of Fe(II) is bound to a histidine side chain of the globin protein, while the sixth site is available to molecules affecting color of the product. In its reduced form, the oxygen molecule is bound to the heme group of myoglobin ($Mb{-}Fe^{2+}{-}O_2$) resulting in flesh that has a bright red color. However, dark muscle tissue of tuna is susceptible to autoxidation (*11*) upon cutting and freezing, forming metmyoglobin ($Mb{-}Fe^{3+}{-}H_2O$), which is brown in color (*12*). Treatment of tissue with CO

* Author to whom correspondence should be addressed [telephone (425) 483-4939; fax (425) 483-4918; e-mail cindy.wu@fda.hhs.gov].

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**Table 1.** Tuna and Mahi-Mahi Acquisition and Description

| sample | store | product name | form | color (visual observation) | origin |
|---|---|---|---|---|---|
| 1 | A | mahi-mahi | fresh | slightly red | Hawaii |
| 2 | B | mahi-mahi | fresh | slightly red | Hawaii |
| 3 | B | mahi-mahi | fresh | slightly red | Hawaii |
| 4 | B | Ahi tuna | fresh | deep red | S. Pacific |
| 5 | C | Ahi tuna | fresh | deep red | Hawaii |
| 6 | B | Ahi tuna | fresh | deep red | Philippines |
| 7 | D | Ahi tuna | frozen/vacuum pack | light brown | Tahiti |
| 8 | C | Ahi tuna | frozen/vacuum pack | watermelon | Indonesia |
| 9 | B | Ahi tuna | frozen/vacuum pack | watermelon | unknown |
| 10 | A | Ahi tuna | frozen/vacuum pack | watermelon | Vietnam |
| 11 | E | Ahi tuna | frozen/vacuum pack | watermelon | Philippines |
| 12 | F | Ahi tuna | frozen/vacuum pack | watermelon | Indonesia |
| 13 | B | Ahi tuna | frozen/vacuum pack | pale pink | Vietnam |
| 14 | F | Ahi tuna | previously frozen | watermelon | unknown |
| 15 | E | Ahi tuna | previously frozen | watermelon | unknown |
| 16 | E | Ahi tuna | previously frozen | watermelon | unknown |

results in the stabilization of color (bright cherry red) due to the greater affinity (>240 times) of CO for the Fe(II) binding site of myoglobin (*2*). Thus, autoxidation and discoloration are prevented when CO is bound, preserving the bright red color associated with tissue that is presumably fresh for an extended period of time.

In the United States, tuna treated with CO or TS must display labeling (21 CFR-§ 101.22j) indicative of that process. Qualitative determination of CO treatment on a product can be determined by persistence of flesh color upon thawing and holding at room temperature (*13*), unnatural color determined by quantitative sensory evaluation (*14*), or colorimeter/image analysis measurements (*15*, *16*). However, these techniques do not consider penetration or total absorption of CO into the muscle tissue. Many spectrophotometric methods have been used to determine various myoglobin derivatives from beef (*17*) and fish muscles (*10*, *18*, *19*) after myoglobin extraction and purification. These methods give quantitative information by measuring amounts of carboxymyoglobin (MbCO) in the muscle tissue. Other researchers developed CO quantitation procedures using GC coupled with flame ionization detector (FID) detection of methane reduced from CO released from fish tissues (*20*−*22*) or blood (*23*). The GC/FID-nickel catalyst approach was employed by the Japanese government in its attempt to regulate imported seafood products (*20*, *21*).

This pilot study describes a direct measurement of CO using gas chromatography/mass spectrometry (GC/MS) following chemically induced CO liberation from fish muscle. Determination of CO using GC/MS for biological samples has been previously reported in forensic application (*24*) and medical research (*25*). The present method is aimed at being simple, rapid, and quantitative. We also feel that detection by mass spectrometry allows confirmation of the compound in question, in this case, CO, and therefore most appropriately fits the potential regulatory need.

## MATERIALS AND METHODS

**Chemicals and Supplies.** Mention of brand or firm names does not constitute an endorsement by the U.S. Food and Drug Administration over others of a similar nature not mentioned. Anhydrous 1-octanol was obtained from Sigma-Aldrich (Milwaukee, WI); concentrated sulfuric acid was obtained from Acros Organics (Pittsburgh, PA); carbon monoxide, 99.5%, was obtained from Liquid Carbonic (Danbury, CT); and headspace vials with screw caps (20 mL), Wheaton media bottles (125 mL), and Wheaton open top screw caps with septum (size 33−430) were obtained from Fisher Scientific (Pittsburgh, PA).

**Standards.** Standards for daily curves were prepared as follows. Carbon monoxide was bubbled into an inverted and submerged 20 mL headspace vial filled with water (held at room temperature, 22 ± 2 °C) until all of the water was displaced by gas. The preparation of a stock vial was then completed by quickly capping and removing the vial from the water. A calibration curve for CO was subsequently established by transferring 1, 2.5, 10, 50, and 150 $\mu$L portions of CO from a stock vial into five capped 20 mL headspace vials using a gastight syringe. An aliquot of 100 $\mu$L from each of the five dilutions was injected into the GC/MS system. Daily temperature and barometric pressure readings were recorded. The actual mass of CO in the standard curves was estimated utilizing the ideal gas law equation, $PV = nRT$, where $P$ is the pressure, $V$ is the volume the CO gas occupies, $n$ is the number of moles of CO present, $R$ is the universal gas constant, and $T$ is the temperature in Kelvin.

**Sample Preparation and Design.** *Acquisition.* Fresh and frozen (vacuum-packed) samples of tuna and mahi-mahi were obtained from local markets (**Table 1**). Vacuum-packed frozen products were placed in a laboratory ultralow freezer (approximately −80 °C) for future analysis. To determine the initial/indigenous CO content of fresh fish tissue, a portion of a single piece of fresh tuna steak or mahi-mahi fillet was analyzed immediately after the product was divided in half. The remaining portion was treated with CO gas as follows.

*In-Laboratory Treatment of Fresh Samples.* Each fresh skinned steak or fillet was vacuum packaged and subsequently exposed to CO by injection into a corner of the packaging until the bag was filled with the gas. Upon resealing, the bag was placed in the refrigerator (4 °C) for 22−24 h to allow CO absorption by fish tissue. Carbon monoxide content of this fresh/CO-treated fish sample was determined at the end of the 22−24 h CO treatment.

*Analysis.* Each individual unit of fish was homogenized in a food processor, and 40 g portions were placed in Wheaton media bottles. Vacuum-packed frozen products were removed from the ultralow freezer and thawed in their original packages before being homogenized. To each aliquot was added 100 $\mu$L of 1-octanol, to suppress foaming, followed by sufficient distilled water to reach the 125 mL mark. The bottles were immediately capped and either shaken vigorously by hand for approximately 30 s or placed on a mechanical shaker for 5 min. Using a separate needle as a vent source, an aliquot of 10 mL of sulfuric acid (5 M) was added via syringe. Upon addition of the acid, the bottles were briefly shaken, placed in a 70 °C water bath for 1 h, and shaken again when removed from the bath. Analysis of CO was completed by injecting 100 $\mu$L from headspace into the GC/MS system after the bottle content cooled to room temperature.

**Gas Chromatography and Mass Spectrometry.** An Agilent 6890N GC (Agilent Technologies, Palo Alto, CA) equipped with an HP-PLOT MOLESIEVE capillary column, 30 m × 0.32 mm i.d. × 12 $\mu$m (J&W Scientific) was used. Oxygen and nitrogen elute earlier than carbon monoxide under the experimental condition described. The samples were manually injected, using a 100 $\mu$L gastight syringe, into the 2

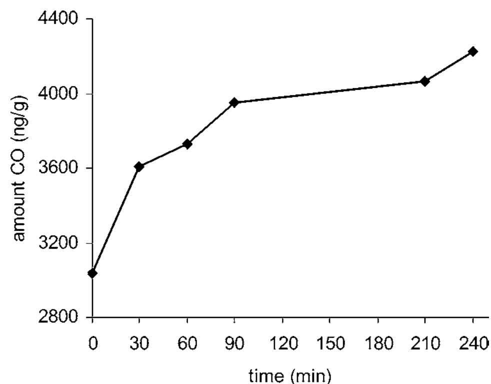


**Figure 1.** Level of CO liberated, via sulfuric acid, from fish flesh homogenate at room temperature.

mm i.d. liner within the injection port set at 120 °C. The oven temperature was held at 30 °C for 2.5 min, ramped to 60 °C over the next 0.5 min, and held at 60 °C for the remaining 2 min of analysis. Helium was the carrier gas and held at a constant flow of 1.5 mL $min^{-1}$. The transfer line was set at 280 °C, and the Agilent 5973 inert mass spectrometer was operated in electron ionization (EI) mode at 70 eV. After a 3.20 min solvent delay, the filament was turned on. Full scans were acquired from *m/z* 10 through 40, or selected ion monitoring at *m/z* 28 was used.

**Safety.** Carbon monoxide is an inflammable and poisonous gas. All preparations involving CO were performed under the hood in a room equipped with CO detectors.

## RESULTS AND DISCUSSION

Previous work (*20*−*22*) demonstrated the ability to determine CO from fish flesh using a packed GC column for separation, methanizer for reduction of CO, and detection by FID. More recently, headspace analysis using GC/MS for CO quantitation was described in forensic and medical research (*24*, *25*). The method reported herein allows for the direct detection and quantitation of CO from fish flesh by GC/MS and was developed with the intent to minimize sample handling. It was determined in the present study that liberating the CO directly from a fish flesh homogenate in water was more effective than releasing the CO from supernatant following extraction of the myoglobin as previously described (*20*−*22*). In addition to eliminating an extraction step, analysis of fish flesh homogenate versus myoglobin containing supernatant revealed CO detection via the former to be 2-fold greater than the latter using a single extraction step (data not shown). Sulfuric acid (*20*, *22*, *23*) of various concentrations and 20% (w/v) potassium ferricyanide (*24*) have both been reportedly effective in liberation of CO from biological samples (e.g., muscle and blood). We compared and determined experimentally that sulfuric acid was superior and less expensive than potassium ferricyanide in the release of CO from tissue homogenate under the conditions stated in the materials and methods section. Among three sulfuric acid concentrations (1, 5, and 10 M) tested, we found 5 M to be most suitable. **Figure 1** shows the amount of CO detected from fish flesh homogenate, over time after the addition of sulfuric acid, while keeping the reaction vials at room temperature. Heating the acidified homogenate at 70 °C for 1 h accelerated the release of CO, thus bringing the reaction and/or release of CO to equilibrium in a timely manner.

We chose to use a molecular sieve column to allow for separation of CO from nitrogen and oxygen, thereby eliminating the need to flush the headspace of each sample bottle with helium prior to CO liberation. The efficacy of this type of column for CO measurement was demonstrated by Leffler et

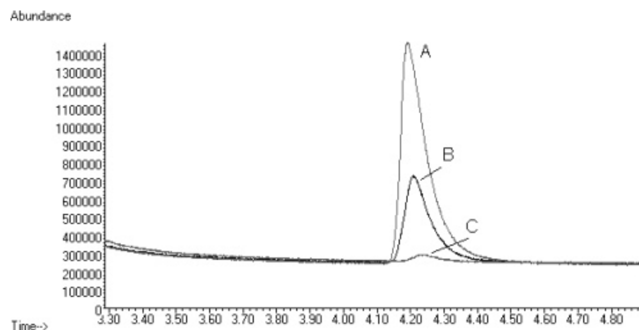


**Figure 2.** Chromatogram of CO peak: (**A**) CO-treated, in-laboratory, fresh tuna; (**B**) CO standard equivalent to 1.8 μg/g; (**C**) untreated fresh tuna.

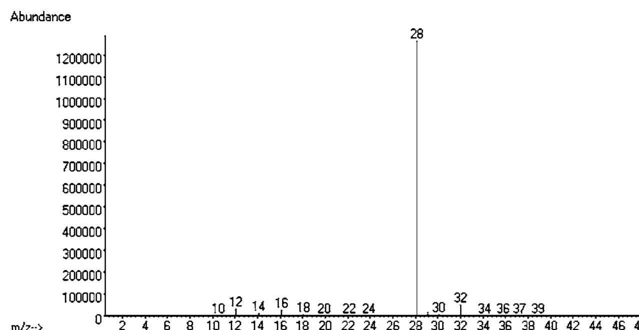


**Figure 3.** Mass spectrum (EI) of CO from treated tuna sample (scan: 4.2 min).

al. (*25*) previously. Optimal separation and peak shape were observed keeping the column temperature at 30 °C for 2.5 min before ramping up to a final temperature of 60 °C (**Figure 2**). An isocratic run at higher temperature decrease separation capacity, while a low-temperature isocratic run caused a very broad CO peak and lower sensitivity. Regardless of the size of the injection, the peak shape was unacceptable when a 4 mm liner was tested in an attempt to load maximum headspace gas onto the column. Satisfactory results were achieved with a 2 mm i.d. inlet liner and a 100 μL injection of headspace gas. For daily analysis, a 3.20 min delay for MS detection was set to help extend filament lifetime. In the EI mass spectrum shown in **Figure 3**, the molecular ion at *m/z* 28 was the base peak and very minor ions were seen at *m/z* 16 and 12. Quantification was consistent whether peak areas were derived from the full scan total ion chromatograms or from extracted mass chromatograms for the molecular ion at *m/z* 28. The GC/MS response for CO appears highly linear. All standard curves, covering the range between 0.006 and 0.85 μg CO per injection, showed correlation coefficients greater than 0.999.

To obtain a baseline CO reading for tuna and mahi-mahi, fresh specimens (i.e., never-been-frozen/non-CO-treated) were purchased from local retail stores and subsequently analyzed (**Table 2**). The indigenous CO contents found in the three tuna steaks were comparable to values reported by researchers in Asia (*20*, *22*) and ranged from 84 to 153 ng/g. The baseline CO levels in the three mahi-mahi samples, ranging between 65 and 95 ng/g, seemed slightly lower than those of tuna, most likely due to the higher myoglobin content in tuna. A separate portion of each fresh sample was treated with CO for 24 h and then homogenized and analyzed for CO. Results for both the tuna and the mahi-mahi reveal a large window between the untreated and treated specimens, 1 order of magnitude or greater difference in CO levels. Slight color changes toward a red flesh were observed during treatment of the mahi-mahi samples. The greatest color change was observed in mahi-mahi samples taken from fillets with the brightest blood line. Although frozen CO-

**Table 2.** Levels of CO in Fresh Mahi-Mahi and Ahi Tuna before and after CO Treatment

| sample | product | CO treatment | mean[a] CO level (ng/g) | RSD, % |
|---|---|---|---|---|
| 1 | mahi-mahi | untreated | 95 | 7.0 |
| 2 | mahi-mahi | untreated | 92 | 2.7 |
| 3 | mahi-mahi | untreated | 65 | 7.9 |
| 1 | mahi-mahi | in-lab, ∼24 h | 943 | 9.9 |
| 2 | mahi-mahi | in-lab, ∼24 h | 1480 | 10.5 |
| 3 | mahi-mahi | in-lab, ∼24 h | 2450 | 4.8 |
| 4 | Ahi tuna | untreated | 103 | 13.3 |
| 5 | Ahi tuna | untreated | 153 | 5.9 |
| 6 | Ahi tuna | untreated | 84 | 15.1 |
| 4 | Ahi tuna | in-lab, ∼24 h | 3351 | 10.4 |
| 5 | Ahi tuna | in-lab, ∼24 h | 2832 | 9.8 |
| 6 | Ahi tuna | in-lab, ∼24 h | 4649 | 2.5 |

[a] Three replicates.

**Table 3.** Levels of CO in Frozen Vacuum-Packaged Ahi Tuna and Mahi-Mahi

| sample | product | labeled as CO-treated | mean[a] CO level (ng/g) | RSD, % |
|---|---|---|---|---|
| 7 | Ahi tuna | no | 162 | 12.8 |
| 8 | Ahi tuna | yes | 1251 | 10.0 |
| 9 | Ahi tuna | | 890 | 10.0 |
| 10 | Ahi tuna | yes | 2533 | 10.7 |
| 11 | Ahi tuna | yes | 1743 | 6.6 |
| 12 | Ahi tuna | yes | 2446 | 3.0 |
| 13 | mahi-mahi | yes | 506 | 11.7 |

[a] Three replicates.

treated tuna has a distinct watermelon hue, there was no color change observed during the treatment of the fresh tuna samples, as they were already blood red in color.

A survey of frozen products obtained from local grocers affirms the results found during the in-lab treatment of fish samples (**Table 3**). The popularity of CO-treated tuna product in the U.S. market has increased significantly over the past decade mainly due to its bright-red color preferred by many consumers. This bright red color has been associated with “freshness” by many consumers although not scientifically true. Many factors, including the fat content, actual species and cut, determine the color of a piece of tuna. The industry is well aware of the fact that tuna meat quickly turns an unappetizing brown, whether it is fresh or conventionally frozen and thawed. In fact, it is empirically known that tuna, once frozen and thawed, could discolor more quickly than unfrozen meat during subsequent storage as the result of conformational changes in tuna myoglobin (*26*). While we were able to obtain commercially CO-treated frozen tuna from multiple sources, we found only one source of frozen tuna steak that had not been treated with CO with dark-brown appearance. In addition, we only found one sample of frozen/vacuum packed mahi-mahi (CO-treated). The untreated frozen tuna sample was found to be in the 150 ng/g range, while the treated samples were near or above 1 $\mu$g/g. The treated mahi-mahi sample was in the 500 ng/g range. All of the treated frozen samples were confirmed as exposed to CO by labeling on either the individually vacuum packed steaks or the outer box in which they were shipped. The significant difference between CO levels found in treated and untreated fish samples makes it possible to determine if a commercially available product has been exposed to CO even if not labeled as such. Further experimentation would have to be conducted to determine the “minimal dose effect”, that is, whether a fish sample can be treated to an extent that allows for the desired color retention without elevating the CO levels to those we are currently finding in CO-treated fish.

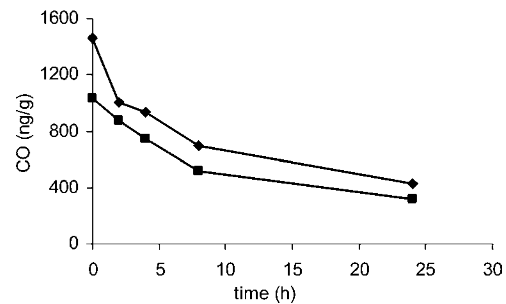


**Figure 4.** Depletion of CO from two commercially treated tuna samples that were homogenized in-lab and subsequently held at 4 °C in an open system. Time zero equals point of homogenization.

**Table 4.** Levels of CO in Previously Frozen Ahi Tuna Purchased from Meat Case

| sample | age (days)[a] | labeled as CO-treated | mean[b] CO level (ng/g) | RSD, % |
|---|---|---|---|---|
| 14 | 1 | yes | 985 | 3.3 |
| 15 | 1 | yes | 1921 | 5.9 |
| 16 | 4 | yes | 1247 | 3.7 |

[a] Number of days stored from packaged on/purchase date. [b] Three replicates.

It is a common practice to remove CO-treated/vacuum-packed frozen tuna steaks from the original packages before display directly on ice or repackage in over-wrap packs for retail sale at refrigeration temperature. We therefore were concerned whether significant loss of CO might occur once the frozen steaks were exposed to atmosphere and began thawing as it might lead to ambiguity from the regulatory aspect. We have found that upon homogenizing the fish in a food processor, it is important to place the flesh in a closed system immediately to prevent CO loss. **Figure 4** displays the loss of CO from fish homogenate held a 4 °C over the course of 24 h. In addition to physically damaging the protein and increasing the surface area of the meat exposed to the atmosphere, homogenization also damages cellular compartments allowing for the release of enzymes and other chemicals. Any or all of the above factors could explain the high rate of loss of CO in fish homogenate. Previously frozen, CO-treated tuna steaks purchased from local grocers’ meat cases, where the tuna steaks had been either left on ice or repackaged in over-wrap packs, were examined to determine if the accelerated loss of CO in homogenate is also observed in steaks left exposed to the atmosphere for 24 h or more (**Table 4**). Carbon monoxide contents of the meat case tuna steaks appear comparable with the levels of CO found in those tuna steaks treated with CO in our laboratory (**Table 2**), indicating that the main concern over loss of CO should focus on closing the system immediately after homogenization.

In conclusion, the above method is potentially useful for the determination of carbon monoxide in fish tissue in conjunction with other methods such as sensory assessment. All of the frozen, commercially CO-treated tuna samples, which had a characteristic watermelon color, were found to contain CO concentrations near or above 1 $\mu$g/g in the present study. Fresh or frozen untreated tuna samples had levels near or below 150 ng/g. The wide gap in values obtained for CO-treated versus untreated tuna and mahi-mahi suggests the easy chemical confirmation of CO treatment using modern and available analytical instrumentation if needed.

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