# Canned bluefin tuna, an in vitro cardioprotective functional food potentially safer than commercial fish oil based pharmaceutical formulations

Gian Carlo Tenore [a,*], Giorgio Calabrese [a], Alberto Ritieni [a], Pietro Campiglia [b], Daniela Giannetti [a], Ettore Novellino [a]

[a] Department of Pharmacy, Università di Napoli Federico II, Via D. Montesano 49, 80131 Napoli, Italy
[b] Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Via Ponte Don Melillo, 1, 84084 Salerno, Italy

## ABSTRACT

Commercial canned fish species typical in the Italian market were evaluated for their lipid profile. Bluefin tuna samples showed the highest content in omega-3 fatty acids (*n*-3 PUFA) among the canned fish samples analyzed. Tests on H9C2 cardiomyocytes revealed that bluefin tuna *n*-3 PUFA may responsible for a significant cell protection against both physiological and doxorubicin-induced oxidative stress. Analogous tests performed by incubating cardiac cells with *n*-3 PUFA ethyl esters, of which most of fish oil pharmaceutical formulations (FOPF) are based, showed cytotoxicity at high doses. Our results highlighted that *n*-3 PUFA contents in a 50 g canned bluefin tuna portion would be almost equivalent to and potentially safer than those of 1 FOPF capsule (1000 mg)/die usually suggested for hyperlipidaemic subjects. Thus, Italian commercial canned bluefin tuna could be indicated as a functional food with potential health benefits for the prevention and care of cardiovascular disorders.

© 2014 Elsevier Ltd. All rights reserved.

## 1. Introduction

The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) establishes that food is "functional" whether its positive effects on one or more organism functions are clearly proved so to be determinant for optimizing health conditions and/or decreasing disease incidence, independently from its nutritional capacity. Moreover, functional food should exert its potential bioactivities through quantities usually planned in a conventional dietary regimen (Diplock et al., 1999). Functional foods can be divided into "conventional" and "modified". The first category consists in foods whose bioactive components are original constituents. The second category includes foods whose biological potential has been technologically influenced through: addition of bioactive compounds; removal of antinutrients or toxic substances; replacement of original components with exogenous

bioactive ones; improvement of the bioavailability of original bioactive constituents.

The correlation between fish consumption and reduction of cardiovascular disease risk has focused scientific research attention since the seventies of the last century. The protective effects can be appreciated even when consumption levels are not elevated: a dietary regimen including at least 30 g fish/day can promote a significant prevention against cardiovascular disorders; particularly, an increase of 20 g/day in fish consumption would lead to a decrease by 7% of death risk for cardiovascular disease in subjects who occasionally consume fish (Mozaffarian et al., 2003). Although the biochemical mechanism of these beneficial properties have not been clearly explained so far, the positive effects of fish consumption would be mainly correlated to fish content in omega 3 long chain polyunsaturated fatty acids (*n*-3 PUFA), particularly, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), of which fish represents the main dietary source for man (Arino et al., 2005). The levels of these two fatty acids are strongly variable, both among the different fish species and within the same species, due to the type of diet and breeding. DHA and EPA are not directly produced by fish organism; they derive from unicellular algae occurring in the food chain (Arterburn et al., 2006). Assumption levels of DHA and EPA negatively correlate with the incidence of

degenerative diseases, such as cardiovascular, neurodegenerative and immune disorders. It has been reported that *n*-3 PUFA intake can reduce triglyceride plasma levels and platelet aggregation, and stabilizes the cardiac rhythm (Harris, 2009). Typical Western diet provides relatively small amounts of *n*-3 PUFA, probably inadequate to protect the body from chronic degenerative diseases. This deficit has been indicated as one of the main causes of the growing incidence of chronic diseases in our society. According to the American Heart Association Nutrition Committee, two servings of fish per week should be consumed for the prevention of cardiovascular diseases (Kris-Etherton et al., 2002). Previous studies have revealed that EPA and DHA would exert their best activity through a synergistic action and that a EPA/DHA ratio of 0.9:1.5 would be adequate for ideal cardiovascular protection (Mori and Woodman, 2006). In fact, this is precisely the respected ratio in most of pharmaceutical formulations based on fish oil *n*-3 PUFA extracts and indicated for the control of serum triglycerides in hyperlipidaemic subjects or for the secondary prevention in patients with previous myocardial infarction.

In the Western world, it is frequently overlooked that fish often means canned fish. Tuna, mackerel, sardines, salmon, and so on, reach our tables mainly as canned products, keeping intact most of their nutritional characteristics in quality controlled and long shelf-life packaging, which greatly facilitate, even in areas not close to the sea, the possibility of ideal fish consumption levels. Nevertheless, the biological potential of canned products can be significantly different respect to raw fish mainly due to thermal processing (fish steam-cooking and, then, can sterilization). Particularly, the deleterious effects of high temperatures on canned fish *n*-3 PUFA, mainly DHA and EPA, have been previously reported (Stephen et al., 2010; Miklavčič et al., 2011). Thus, the aim of the present work was to analyze the lipid profile of the most common canned fish species brands in the Italian market in order to evaluate their potential health benefits. The attention was focused on the products canned in brine considered as the closest to raw fish, particularly as regards their lipid profile, if compared to the ones canned in edible oils.

## 2. Materials and methods

### 2.1. Reagents and standards

All chemicals and reagents used were either analytical-reagent or GC grade. All organic solvents were purchased from Carlo Erba, Milano (Italy). Water was purified by a Milli-Q$_{plus}$ system from Millipore (Milford, MA) before use. The standard mixture of fatty acid methyl esters, the pure standards *cis*-4,7,10,13,16,19-Docosahexaenoic acid (DHA, $\geqslant$98%) and *cis*-5,8,11,14,17-Eicosapentaenoic acid (EPA, $\geqslant$99%), were purchased from Sigma Chemicals (Milan, Italy).

### 2.2. Sample collection and lipid extract preparation

Fifty-one cans different fish species of various common Italian brands were obtained from retail trades. For each fish species, different numbers of brands were chosen as follows: seven, for *Thunnus thynnus* (bluefin tuna); six, for *Thunnus albacares* (yellowfin tuna); eight, for *Thunnus alalunga* (albacore); seven, for *Scomber scombrus* (Atlantic mackerel); five, for *Engraulis encrasicolus* (anchovy); six, for *Sardina pilchardus* (sardine); five, for *Xiphias gladius* (swordfish); seven, for *Salmo salar* (salmon). For each brand, six lots were taken into consideration. All of the samples had been canned in brine.

Aliquots (5 g) of each sample were freeze-dried and stored at −20 °C until analysis. Around 0.5 g of powder samples were subjected to lipid extraction according to AOAC method 948.16, by using a 6-place units Extraction Unit E-816 Soxhlet (Buchi, Flawil, Switzerland). After centrifugation at 3000*g* for 5 min, supernatants were transferred into a pre-weighed scintillation vial, and dried under nitrogen.

### 2.3. Analysis of fatty acid composition

Lipid extracts (0.1 g) were dissolved in 2 mL of *n*-eptane and treated with 0.2 mL of 2 N potassium hydroxide methanolic solution (11,2 g of potassium hydroxide in 100 mL methanol). The mixture was shaked energically for 1 min at room temperature and then centrifuged (3000*g* for 5 min). Supernatants were

collected and analysis of fatty acid methyl esters was performed by gas chromatography using a DANI GC instrument (DANI Instruments, Milan, Italy) coupled to a flame ionization detector (FID) and equipped with a HP-5 capillary column (Agilent, Milan, Italy). The temperature programme started at 150 °C (10 min), increased by 2 °C/min to 180 °C and then increased again by 3 °C/min to 240 °C (20 min).

### 2.4. In vitro tests

#### 2.4.1. Preparation of samples for in vitro tests

A mixture of commercial DHA and EPA standards was prepared simulating the same relative proportions as in canned bluefin tuna lipid profile, and has been referred to as SBTDEM (Simulated Bluefin Tuna DHA and EPA Mixture).

A typical commercial fish oil based pharmaceutical formulation (FOPF) indicated for the control of plasmatic lipid levels was chosen for the in vitro tests. It consisted in 1000 mg capsules containing PUFA ethyl esters with 85% minimum levels of EPA and DHA (ratio 0.9–1.5) and the following excipients: D,L α-tocopherol, gelatin succinate, glycerol, ethyl *p*-oxybenzoate, propyl *p*-oxybenzoate. Lipid extraction was performed as described in Section 2.2.

#### 2.4.2. Cell culture and viability test

Rat cardiac H9C2 cells (ATCC, Manassas, VA) were cultured (17–21 passages) in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in 150 cm$^2$ tissue culture flasks at 37 °C in a humidified atmosphere of 5% $CO_2$. The cells were fed every 2–3 days, and subcultured once they reached 70–80% confluence. Cell viability and proliferation were assessed by incubating the culture with SBTDEM and FOPF lipid extract samples (0.01–2 μg), and doxorubicin 1 μM for 72 h. Lipid extract samples were solubilized by adding 150 μL of DMSO and by mixing it in an orbital shaker for 5 min. As a control, 0.5% DMSO was added to untreated cells.

#### 2.4.3. Preparation of cell extract

Cardiac H9C2 cells were collected by centrifugation and then resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM EDTA. The cells were sonicated, followed by centrifugation at 13,000*g* for 10 min at 4 °C. The resulting supernatants were collected and kept on ice for immediate measurements, as described below.

#### 2.4.4. Measurement of intracellular ROS accumulation

2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA, 5 μM) was used to detect intracellular ROS levels in H9C2 cells (Vanden Hoek et al., 1997). DCF-DA is cell membrane permeable. Once inside the cells, DCF-DA is hydrolyzed by cellular esterases to form DCF, which is trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular ROS to form the fluorescent product, 2′,7′-dichlorofluoroscein. Then, the cells were washed once with phosphate buffered saline (PBS) and lysed in 3 mL ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 0.2% sodium dodecyl sulfate. The cell lysates were collected and centrifuged at 2000*g* for 5 min at 4 °C. The fluorescence of the supernatants was measured using a Perkin–Elmer luminescence spectrometer (LS50B) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

#### 2.4.5. Measurement of cellular superoxide dismutase activity

Total cellular superoxide dismutase (SOD) activity was measured as follows (Kirshenbaum and Singal, 1992). Briefly, a reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/mL catalase, 70 μM nitroblue tetrazolium, 0.2 mM xanthine, 50 μM bathocuproinedisulfonic acid, and 0.13 mg/mL bovine serum albumin (BSA). A 0.8 mL aliquot of the reaction mixture was added to each cuvette, followed by addition of 100 μL of lysate. The cuvettes were pre-warmed at 37 °C for 3 min. The formation of formazan blue was monitored at 560 nm, 37 °C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma) standard curve, and expressed as units per mg of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA) based on the method which makes use of BSA as the standard.

#### 2.4.6. Measurement of caspase-3 activity

Caspase-3 activity was measured using the BD ApoAlert Caspase-3 Fluorogenic Assay (BD Biosciences Clontech, Palo Alto, CA). Briefly, protein lysates were collected from cells that had been incubated with lioRGJ (0.01–1 μg) for 8 h, as per protocol. Activity was measured using a fluorescent microplate reader (PerSeptive Biosystems, Farmington, MA).

### 2.5. Statistics

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student's *t* test or two-way ANOVA followed by