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F₃-Isoprostanes as a measure of *in vivo* oxidative damage in *Caenorhabditis elegans*

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Abstract

Oxidative stress has been implicated in development of a wide variety of disease processes, including cardiovascular disease, cancer, and neurodegenerative diseases, as well as progressive and normal aging processes. Isoprostanes (IsoPs) are prostaglandin-like compounds that are generated *in vivo* from lipid peroxidation of arachidonic acid (AA, C20:4, ω -6) and other polyunsaturated fatty acids (PUFA). Since the discovery of IsoPs by Morrow and Roberts in 1990, quantification of IsoPs has been shown to be excellent biomarkers of *in vivo* oxidative damage. Eicosapentaenoic acid (EPA, C20:5, ω -3) is the most abundant PUFA in *C. elegans* and gives rise to F₃-IsoPs upon nonenzymatic free radical-catalyzed lipid peroxidation. The protocol presented is the current methodology that our laboratory uses to quantify F₃-IsoPs in *C. elegans* using gas chromatography/mass spectrometry (GC/MS). The methods described herein has been optimized and validated to provide the best sensitivity and selectivity for quantification of F₃-IsoPs from *C. elegans* lysates.

Keywords

C. elegans; F₃-Isoprostanes; oxidative stress; lipid peroxidation; reactive oxygen species

INTRODUCTION

Lipid peroxidation is a hallmark of oxidative stress, in part due to its susceptibility to free radical attack^{1,2}. Damage to tissue biomolecules, including lipids, proteins and DNA by reactive oxygen species (ROS) is thought to be a major contributor to pathophysiology of oxidative stress^{3–5}. ROS are chemically reactive, oxygen-containing molecules typically generated during aerobic metabolism, with the mitochondria being a major source of endogenous ROS^{6,7}. Some of the most common ROS produced in living systems are superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (•OH). Reactive nitrogen species (RNS) are related reactive species formed in cells, which include

peroxynitrite (ONOO⁻), nitric oxide (·NO) and other carbon-centered radicals. Excess ROS accumulation can cause injury to surrounding biomolecules; therefore the cell has developed a series of antioxidant defense mechanisms to mitigate the effects of free radical-induced oxidative damage and maintain redox homeostasis. The primary antioxidant defenses include antioxidant enzymes to degrade ROS, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, along with low molecular weight antioxidant molecules to serve as free radical scavengers and/or reducing agents. Oxidative stress occurs when there is an imbalance between production of free radicals and antioxidant defenses to favor an environment where excess ROS can cause oxidant damage to biomolecules.

Measuring oxidative stress requires accurate quantification of either free radical production or damaged biomolecules, such as lipid peroxidation products. Several analytical approaches [electron paramagnetic resonance (EPR), chemiluminescence, fluorescence] have been used to detect levels of intraworm ROS and their oxidation products; however many of these techniques suffer from lack of sensitivity and specificity, as well as being methodologically difficult to execute. In a recent multi-investigator study sponsored by the National Institutes of Health (NIH), the Biomarkers of Oxidative Stress Study (BOSS) found that the most accurate method to assess oxidant stress *in vivo* is through quantification of plasma or urinary F₂-Isoprostanes (F₂-IsoPs)⁸. The predominant mammalian polyunsaturated fatty acid, arachidonic acid (AA), can give rise to non-enzymatic formation of F₂-IsoPs through free radical-induced lipid peroxidation⁹. Because of their chemical stability and prevalence in all human tissues and biological fluids, IsoPs are regarded as the gold standard for quantification of oxidative damage in mammals. In *C. elegans* the major fatty acid eicosapentaenoic acid (EPA) forms F₃-IsoPs, which were also recently identified as markers for oxidative damage¹⁰⁻¹².

This unit will describe methods to measure F₃-IsoPs in *C. elegans* using a gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) approach employing a stable isotope dilution. This includes solid-phase extraction (SPE), thin-layer chromatography (TLC) purification, chemical derivatization, and MS detection using negative ion chemical ionization (NICI) coupled with GC. The advantages of GC-MS include the high resolution of GC separation on fused silica capillary columns and the specificity and sensitivity of MS, which allows the lower limit of detection of the F₃-IsoPs to be in the low pictogram range. This assay is a robust and sensitive method that enables quantification of endogenous levels of oxidative damage in *C. elegans*. A goal of this unit is to introduce a sensitive and accurate measure of endogenous oxidative damage in *C. elegans* for oxidant stress studies. Several considerations of this protocol and alternative approaches are discussed in the Commentary section below. Methods are described for collection of whole worm lysates (Basic Protocol 1), lipid extraction for measurement of total F₃-IsoPs (Basic Protocol 2), and sample preparation with chemical derivatization and extraction for GC-MS analysis (Basic Protocol 3).

BASIC PROTOCOL 1

SAMPLE COLLECTION AND PREPARATION

This protocol describes the method for nematode collection and homogenization for quantification of F₃-IsoPs as a measure of lipid peroxidation. A critical consideration in the accurate measurement of oxidant damage is decreasing the *ex vivo* oxidation of eicosapentaenoic acid, which can generate F₃-IsoPs outside of the desired experiment. This may be addressed by flash-freezing washed samples in liquid nitrogen directly following collection if not used for sample preparation and analysis immediately.

Materials—NaCl

1× Tris-buffered saline, pH 7.4 (Fisher, acidify to pH 6.8 with HCl)

1× Protease Inhibitor Cocktail (Sigma, cat. no. P8465)

1% Triton X-100 (Sigma, cat. no. T8787)

2-ml sterile polypropylene screw-cap tubes

1.0 mm zirconia beads (BioSpec Products, cat. no. 11079110zx)

High-energy cell disruptor, (BioSpec Products, cat. no. 607)

Worm collection

- 1 Harvest healthy synchronized nematodes by washing plates with room temperature 85 mM NaCl, using 10–12 mL per plate. Transfer resuspended nematodes to 15 mL conical tubes using sterile pipettes and centrifuge to pellet worms for 1 minute at 400 × *g*, 23°C. Repeat washes at least 3 times or until aqueous phase is clear.
- 2 Centrifuge worms at 1600 × *g*, 23°C, for 5 minutes. Aspirate the supernatant down to 100 µL and transfer worm solution into 2-mL polypropylene microvials.

Preparation of homogenization solutions

- 3 Prepare lysis buffer: (0.0497 g NaCl, 100 µL of 1% Triton X-100, 100 µL of 1M Tris buffered saline, pH 6.8, 100 µL of 1× protease inhibitor cocktail, and 9.7 mL of sterile water). Aliquot 2 mL into 15 mL conical tubes and store at –20 °C. Keep aliquot of freshly prepared lysis buffer in an ice bucket for immediate use.
- 4 To prepare samples for homogenization, add 400 µL cold lysis buffer to 2-mL polypropylene microvial containing nematodes.

Eicosapentaenoic acid in tissue samples can oxidize *ex vivo* to generate F₃-IsoPs. If worm samples are not to be used immediately after collection, flash freeze the sample in liquid nitrogen and store at –80 °C.

- 5 To add zirconia beads to sample vials, cut the tip off the plastic twist-top for easier pouring. Add 300 μ L 1.0 mm zirconia beads into samples for homogenization.
- 6 Bring total lysate volume of each sample to ~700 μ L by adding 200 μ L cold 85 mM NaCl. Prepare stirring ice water on stir plate to cool samples in between homogenization.

Homogenization of nematodes

- 7 To homogenize worms, all following steps should be performed in a 4 °C cold room. Homogenize worms with a beadbeater at full speed for 20 s. Remove samples from beadbeater and place in stirring ice water for 1 min. Repeat homogenization and cooling steps for a total of 7 cycles.
- 8 Centrifuge for 30 min at $3500 \times g$ in a tabletop centrifuge at 4 °C to separate aqueous and organic layers. After centrifugation, carefully pipette off aqueous layer and transfer to a new 2-mL polypropylene microvial. Lysates can be stored at -80 °C until ready for sample purification.

Cleaning of zirconium beads

- 9 Transfer used zirconium beads from sample tubes into a clean, open stainless steel or glass tray.
 - 10 Soak beads in diluted bleach mixture (1:10 bleach:H₂O) for 5 minutes. Rinse beads in distilled water until aqueous phase is clear.
 - 11 Dry beads in glass tray at 40 – 70C oven. Beads can be autoclaved for 30 minutes and stored in dispensing container before use.
- Zirconium beads can be reused for approximately 10 uses before beads wear down in size.

BASIC PROTOCOL 2

LIPID EXTRACTION AND HYDROLYSIS OF F₃-IsoP-CONTAINING PHOSPHOLIPIDS IN *C. elegans* LYSATES

F₃-IsoPs are formed *in situ* esterified to phospholipids and are subsequently released by phospholipases. In tissue samples and worm lysates, this phenomenon creates two forms of F₃-IsoPs; one form that remains esterified in the membrane, and a second that is hydrolyzed and released in free form. Both free and esterified F₃-IsoPs are quantified for total F₂-IsoP formation in the worm lysates. In order to quantify total F₃-IsoPs, it is necessary to extract the phospholipids from the tissue and release esterified F₃-IsoPs from the phospholipids via base hydrolysis. A free radical scavenging agent, BHT, is added to the organic solvent during extraction of phospholipids in order to decrease the amount of *ex vivo* oxidation of EPA.

Materials—15% KOH, wt/v (see recipe)

Methanol containing BHT (0.005% v/v) (see recipe)

pH 3 water (see recipe)

1 N HCl

1.7-ml microcentrifuge tube

15-mL plastic tube (Denville, cat. no. T8173)

1. Let samples thaw on ice. Once thawed, aliquot 30 μ L of lysate into a new 1.7-mL microcentrifuge tube for protein content normalization.
2. To each sample add 500 μ L of KOH (15%, wt/v). Mix gently by inversion, making sure to not vortex samples and incubate at 37 °C for 30 min.

To quantify total F₃-IsoPs formation, both free and esterified F₃-IsoPs are analyzed by releasing F₃-IsoPs from the phospholipids via base hydrolysis.

3. Add 500 μ L of methanol containing butylated hydroxytoluene (BHT) to each sample.

Adding a free radical scavenging agent like butylated hydroxytoluene (BHT) to the lipid extracts is important to inhibit ex vivo formation of F₂-IsoPs. Total lipids are dissolved in 500 μ L methanol containing BHT (0.005% v/v) to saponify lipid extracts and release esterified isoprostanes.

4. Mix gently by inversion and centrifuge for 2 min at maximum speed in a tabletop centrifuge at room temperature.

Following centrifugation, a semisolid protein layer should have formed between the upper (aqueous) and the lower (organic) layers.

5. Carefully pipette off semisolid protein layer and discard. Transfer aqueous layer to a 15-mL plastic tube by pipet and place on ice.
6. Acidify the mixture to pH 3 with 1 ml HCl (1N) and bring total volume to 10 mL with pH 3 water in preparation for purification of F₂-IsoPs with solid-phase extraction (SPE).

It is important that samples are corrected to pH 3 or slightly lower (down to pH 2.5) in order for the carboxylate moiety to be protonated in order to chromatograph correctly on the Sep-Pak cartridges. Note, it is important to continue immediately with the purification steps below. The sample should not be stored in this form because of the potential for oxidation.

BASIC PROTOCOL 3

SAMPLE PURIFICATION FOR MASS SPECTROMETRIC ANALYSIS

This GC – MS method is based on those described by Morrow, Roberts, and colleagues^{11,13,14} and involves derivatization of carboxyl moieties on the F₃-IsoPs to the pentafluorobenzyl ester, trimethylsilyl ether derivatives. Separation of compounds is

achieved by solid-phase extraction using both C18 and silica Sep-Pak to remove the contaminants. Pentafluorobenzyl (PFB) moiety is then reacted with the molecule to enhance detection using electron capture chemical ionization techniques¹⁵. Lastly, trimethylsilyl (TMS) is introduced to add to cap the hydroxyl groups to lower its boiling point for ease of vaporization in the gas chromatograph. Analysis of the derivatives of 15-F_{3t}-IsoPs and the internal standard is carried out using the selective-ion monitoring (SIM) techniques, where the ions monitored are *m/z* 567 for F₃-IsoPs and *m/z* 571 for the internal standard, respectively.

Materials—[²H₄]-15-F_{2t}-IsoP (8-iso-PGF_{2α}) (Cayman Chemical, cat. no. 316351)

Methanol (Fisher, cat. no. A4544)

pH 3 Water (see recipe)

1 N HCl

Heptane (Fisher, cat. no. H3504)

Ethyl acetate (Fisher, cat. no. E1964)

Acetonitrile (Fisher, cat. no. A9984)

10% Pentafluorobenzyl bromide (PFBB) (Sigma, cat. no. 10105-2, see recipe)

10% *N,N'*-Diisopropylethylamine (DIPE) (Sigma, cat. no. D3887, see recipe)

Calcium hydride (Sigma, cat. no. 208027)

Chloroform (VWR, EM-CX1059-1)

Ethanol, absolute, 200 proof (Fisher, AC61509040)

10-ml Disposable plastic syringe (Laboratory Supply, cat. no. SMJ5512878)

15-ml plastic tube (Denville, cat. no. T8173)

5-ml plastic vial with cap (Denville, cat. no. T8200)

C₁₈ Plus solid-phase extraction (Sep-Pak) cartridge (Waters Associates, cat. no. WAT036575)

Silica Plus solid-phase extraction (Sep-Pak) cartridge (Waters Associates, cat. no. WAT036580)

Supelco Visiprep SPE vacuum manifold (Sigma, cat. no. 57265)

Analytical evaporation unit with water bath at 37°C (Organomation, cat. no. 11634-P)

TLC filter paper (VWR, cat. no. 28298-020)

TLC silica plates (Analtech, cat. no. 43931-2)

Glass TLC tank (VWR, cat. no. 21432-761)

Sample purification using Separation-Phase Extraction (SPE)

- 1 To sample accurately add 248 pg of the internal standard ($[^2\text{H}_4]$ -15-F₂t-IsoP) and vortex.
- 2 For reverse phase, connect one C-18 Sep-Pak cartridge to a 10-ml disposable syringe per sample and place on vacuum manifold. Precondition each cartridge with 5 ml methanol and 5 ml pH 3 water.

Use a pressure < 5 mm Hg for column conditioning.
- 3 Dispense acidified sample to the preconditioned Sep-Pak cartridge.

During sample loading, the vacuum manifold should be set at < 5 mm Hg for a flow rate through the column at 1–2 ml per min, such that individual drops emerge from the Sep-Pak. This is to avoid loss of IsoPs when applying sample to the cartridge.
- 4 Wash cartridge first with 5 mL pH 3 water followed by 5 mL heptane.

Heptane removes nonpolar contaminants including un-oxidized EPA.
- 5 Elute F₃-IsoPs with 10 mL of ethyl acetate/heptane (50:50 v/v) into a 15-mL plastic tube.
- 6 Dry ethyl acetate/heptane eluate with anhydrous sodium sulfate. Add 1–5 g Na₂SO₄ to vial and swirl gently for 5 s.

This step removes residual water from the eluent.
- 7 Remove used C-18 Sep-Pak cartridges from syringes and discard. Connect one silica Sep-Pak cartridge to each syringe per sample and place on vacuum manifold. Precondition each silica cartridge with 5 mL ethyl acetate/heptane (50:50 v/v).
- 8 Apply eluent from C-18 Sep-Pak cartridge to silica Sep-Pak cartridge.

Care should be taken to decant eluent and not to transfer any sodium sulfate to the extraction cartridge.
- 9 Wash cartridge with 5 mL ethyl acetate, then elute F₃-IsoPs from the silica Sep-Pak cartridge with 5 mL ethyl acetate/methanol (45:55 v/v) into a 5-mL collection vial.
- 10 Evaporate eluent under nitrogen in the analytical evaporation unit in a fume hood.

Conversion of F₃-IsoPs to corresponding pentafluorobenzyl esters

- 11 Add 40 µL of 10% (v/v) PFBB in acetonitrile and 20 µL of 10% (v/v) DIPE in acetonitrile to the sample vial, vortex briefly, and incubate for 20 min at 37 °C.

Convert F₃-IsoPs to the corresponding pentafluorobenzyl esters. This derivatization facilitates compound analysis by GC/MS. Do not work outside of a well-ventilated fume hood due to PFBB being a potent lachrymator.

- 12 Dry sample thoroughly under nitrogen in an analytical evaporation unit and resuspend material in 50 μ L methanol/chloroform (3:2, v/v). Vortex briefly.

Thin-Layer Chromatography (TLC)

- 13 Prepare TLC tank by adding 92 mL chloroform, 8 mL ethanol, 1 mL acetic acid, and TLC filter paper to saturate the tank. Allow tank to equilibrate for 30 min.
- 14 Mark solvent front line and label sample lanes. Silica plates contain a pre-adsorbent zone towards the bottom of the plate and four pre-scored lanes. Measure 13 cm from top of the pre-adsorbent zone (origin line) and mark. Lightly score solvent front across plate.

Above marked solvent front, label individual samples per each four lane.

- 15 Apply mixture from each sample to a silica TLC plate. Dry samples for 5–10 s with a hair dryer.

Samples are applied to the upper half of pre-adsorbent zone in four pre-scored lanes, taking care to apply only one sample per lane. Avoid adding sample to the first 1 cm of the plate.

- 16 Using a separate TLC plate, apply approximately 2–5 μ g of the methyl ester of prostaglandin F_{2 α} (PGF_{2 α}) to one lane for use as a standard.
- 17 After ensuring that the application solvent has dried, place TLC plates into the TLC tank. Solvent is allowed to migrate 13 cm, and plates are removed from tank for solvent to evaporate.

Not all TLC plates may reach 13 cm at the same time.

- 18 Visualize the TLC standard by spraying the standard plate with the phosphomolybdic acid solution and then heating on a hot plate.

Do not spray sample plates.

- 19 Scrape silica from the sample TLC plates in the region of the TLC standard (R_f should be approximately 0.18), scraping from 0.5 cm above the middle of the visualized standard to 1 cm below the standard.

Keep sample plates until GC/MS analysis is complete.

- 20 Place scraped silica from each sample into separate 1.7-mL microcentrifuge tubes and add 1 mL ethanol in ethyl acetate (15%, v/v). Vortex vigorously for 10–30 s to extract analytes from the silica, then centrifuge in a table-top centrifuge at maximum speed (126,600 $\times$ g) for 2 minutes at room temperature.
- 21 Carefully transfer ethanol in ethyl acetate and place in a new 1.7-mL microcentrifuge tube.

Silica may affect instrument sensitivity; therefore take care not to disrupt the silica pellet in the bottom of the tube.

- 22 Dry under nitrogen in the analytical evaporation unit in a fume hood.

Formation of trimethyl silyl ether derivatives

- 23 Once dried, dissolve samples in 20 μ L bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 6 μ L dry dimethylformamide (DMF).

This reaction converts analytes to the trimethylsilyl ether derivatives.

- 24 Vortex well and incubate sample at 37 °C for 5 min.
- 25 Dry reagents under nitrogen in the analytical evaporation unit.

Quantification of F₃-IsoPs by GC-MS

- 26 Resuspend sample in 20 μ L dry undecane and vortex briefly. Transfer sample to an autosampler vial for GC/MS analysis.
- 27 S GC-NICI-MS approach employing stable isotope dilution will be utilized for F₃-IsoP quantification. The major ions generated in the NICI mass spectra of the pentafluorobenzyl ester, tris(trimethylsilyl) ether derivatives of F₃-IsoP are m/z 567 and corresponding ion for the [²H₄]15-F₂I-IsoP internal standard, m/z 573.
- 28 To quantify, the height of the peak containing the derivatized F₃-IsoP is compared to the height of the deuterated internal standard peak. Levels of F₃-IsoPs in nematodes are reported in nanograms per gram of total protein content.

REAGENTS AND SOLUTIONS

85 mM NaCl—10 g NaCl

2 liter H₂O

Autoclave 45 min

Store up to 1 year at 4°C

15% wt/v KOH—15 g KOH

100 ml H₂O

Store up to 1 year at 23°C

Methanol containing BHT (0.005% v/v)—5 mg BHT

100 ml methanol

Store up to 1 year at 23°C in an amber-colored bottle

pH 3 Water—500 ml H₂O

500 μ L 1 N HCl

Store up to 1 year at 23°C

10% v/v PFBB—1 ml PFBB

9 ml Dry acetonitrile

Store over calcium hydride to keep solution free of water.

10% v/v DIPE—1 ml DIPE

9 ml Dry acetonitrile

Store over calcium hydride to keep solution free of water.

COMMENTARY

Background Information—Given the amount of evidence implicating oxidative stress in the etiology of cancer, neurodegenerative disease, and aging, studying the role of oxidative damage will broaden knowledge of disease. In order to collect a comprehensive examination of the role of oxidative stress in disease, methods to directly quantify the amount of oxidative damage and ROS generation are in demand.

Fluorescent or chemiluminescent dyes, such as dichlorodihydrofluorescein (DCFH-DA), dihydroethidium, and dihydrorhodamine are used frequently for measuring H_2O_2 , $O_2^{\bullet-}$ and $ONOO^-$ either in whole worm or isolated mitochondria^{16–18}. The most prevalently used fluorescent probe for detecting intracellular H_2O_2 and oxidative stress is DCFH-DA. DCFH-DA is cell-permeable and is hydrolyzed intracellularly to the DCFH carboxylate anion, which is retained in the cell. Two-electron oxidation of DCFH forms the fluorescent product, dichlorofluorescein (DCF), which can be monitored by several fluorescence-based techniques, such as fluorescent microplate readers, confocal microscopy, and flow cytometry. Despite the user-friendly appeal of DCFH-DA dye, there are several limitations and artifacts associated with the DCF assay due to the complex intracellular redox chemistry of DCFH^{19–21}. A serious caveat to consider is the indirect nature of DCF fluorescence; DCF is not formed through the direct reaction between DCFH and H_2O_2 . Therefore, DCF fluorescence is not a direct measure of H_2O_2 levels in the worm. In addition, several oxidizing species will oxidize DCFH to DCF, making the dye nonspecific for a particular form of ROS. And lastly, the intermediate radical, $DCF^{\bullet-}$, rapidly reacts with O_2 to form $O_2^{\bullet-}$. The dismutation of $O_2^{\bullet-}$ yields additional H_2O_2 ²², which can establish a redox-cycling mechanism leading to artificial amplification of fluorescence signal intensity. Therefore, it is important to recognize the limitation of the dyes and avoid erroneous interpretations; DCFH-DA cannot reliably quantify intracellular H_2O_2 and ROS as a measure of oxidative stress.

As previously discussed, real-time quantification of ROS in living organisms is limited with redox-sensitive probes due to the non-specific and disruptive nature of the fluorescent dyes. To overcome the limitation of the dyes, recent advances in constructing genetically encoded

redox-sensitive sensors allows for new avenues for investigating redox signaling^{23,24}. Use of redox sensors via either a hydrogen peroxide sensor protein coupled with a glutathione redox potential sensor (HyPer and Grx1-roGFP2, respectively)²³ or quantitative redox proteomics (OxICAT)²⁴ can overcome limitations of the fluorescent dye to provide quantification of ROS in an intact organism. HyPer and Grx1-roGFP2 are ratiometric biosensors that can be used to determine the oxidized-to-reduced ratios of H₂O₂ and GSSG/2GSH. OxICAT is a redox proteomic technique, which monitors the oxidation status of various redox-sensitive protein thiols with a thiol-reactive affinity tag (ICAT). The use of genetically encoded redox-sensitive sensors and redox proteomics provide efficient methods to study ROS generation.

The discovery of isoprostanes as products of non-enzymatic lipid peroxidation has been a major breakthrough in the field of free radical chemistry. The quantification of F₂-IsoPs and related analogues has extended our understanding the role of free radicals in the physiological processes and has established the occurrence of oxidative stress in a wide variety of disease states. As stated previously, lipids react readily with free radicals, resulting in production of stable peroxidation products. F₂-IsoPs and related analogues form *in situ* in the phospholipid bilayer, which then gets released in free form upon hydrolysis by phospholipases, presumably by the action of phospholipase A₂²⁵ and in part, by platelet-activation factor (PAF) acetylhydrolases I and II²⁶. F₂-IsoPs are stable, robust molecules detectable tissues and biological fluids, including plasma, urine, cerebrospinal fluid, and bile¹⁴. Studies have shown that quantification of isoprostanes in either plasma urine is representative of their endogenous production, therefore measuring lipid peroxidation via IsoP quantification is an accurate index of *in vivo* oxidative stress⁸. As EPA is the most abundant PUFA in *C. elegans*, quantifying the resultant free-radical catalyzed oxidation product of EPA, the F₃-IsoPs, is a highly sensitive and accurate representation of endogenous oxidant damage in the worm.

Numerous approaches have been developed to quantify IsoPs either through use of mass spectrometry^{12,27,28} or immunological approaches²⁹. The methodology described herein uses a gas chromatography – negative ion chemical ionization – mass spectrometry (GC-NICI-MS) approach, where F₃-IsoPs are quantified through measurement of the 15-F_{3t}-IsoP. The advantages of this technique over other methods include its sensitivity, specificity, and reproducibility, with quantitative yields in the low picogram range. One drawback to this particular method is the labor-intensive nature and requirement for considerable equipment expenditures. Several alternative mass spectrometric assays have been developed to quantify F₃-IsoPs; Labuschagne and colleagues developed a liquid chromatography – mass spectrometry assay to measure F₃-IsoPs in *C. elegans*²⁸. An advantage to the LC-MS method is that the sample preparation for LC-MS analysis does not require derivatization of the molecule, and therefore is simpler than sample preparation for GC-MS. One drawback to the LC-MS assay is the higher limits of detection in contrast to the more highly sensitive nature of GC-MS. Other methods to quantify IsoPs involve immunological approaches, employing antibodies against 15-F_{2t}-IsoP, with at least three immunoassay kits available commercially²⁹. The disadvantage of this assay is that precision and accuracy are limited. The sensitivity and/or specificity of these kits might vary substantially between

manufacturers. In addition, currently there is a lack of commercially available immunoassay kits for detecting F₃-IsoPs.

In conclusion, we present a robust GC/MS approach for accurate, sensitive and selective quantification of endogenous oxidative damage in *C. elegans*. The equipment used for purification, derivatization and GC/MS analysis is readily available in most pharmacology laboratories. Robust quantification of F₃-IsoPs can be detected for a wide-range of pathologies affected by ROS, therefore our method should be applicable to studying oxidative damage in the development of pathologies and aging.

Critical Parameters—As F₃-IsoPs can be readily generated *ex vivo* in biological materials containing polyunsaturated fatty acids, it is imperative to take care when the samples are obtained and during processing to prevent *ex vivo* oxidation, which can generate IsoPs. For best results, samples should be flash frozen in liquid nitrogen immediately upon collection and not thawed until sample preparation and analysis. Samples should be stored at -70°C until analysis.

Troubleshooting—For troubleshooting, if the peak signal is extremely low or if no peaks are detected by the mass spectrometer, the sample should be removed from the autosampler vial, dried thoroughly under nitrogen, and steps 6.1 – 7.3 should be repeated. If peaks are still not detected, another likely point of error would be problems with the thin-layer chromatography. Scrape all silica from the bottom half of sample lane, place in a microcentrifuge tube, and extract with ethyl acetate as described in step 5.9. Continue by finishing the assay as described above. If no result is obtained, it will be necessary to repeat the entire analysis with a new sample. If the internal standard is detected with a strong signal at m/z 573 but there are very low or nonexistent peaks at m/z 567, then the level of F₃-IsoPs is below the limit of detection. The limit of detection for this assay is 5 pg.

Anticipated Results—A representative chromatogram obtained from the analysis of F₃-IsoPs in *C. elegans* is shown in Fig. 2. The peaks in upper panel of Fig. 2 represent endogenous F₃-IsoPs in worm lysates. The single peak in the lower m/z 573 chromatogram represents the [²H₄]-15-F_{2t}-IsoP internal standard. For quantification purposes, the height of the peak containing the derivatized F₃-IsoP (m/z 567) is compared to the height of the deuterated internal standard peak (m/z 573). Levels of F₃-IsoPs in whole worm lysates are reported in picograms per microgram of total worm protein.

Time Considerations—In general, 12–15 samples can be assayed in approximately 10 hrs by an experienced investigator. Homogenization, lipid extraction, and hydrolysis of this number of worm samples requires ~3 hrs; Sep-Pak purification takes ~2 hrs; drying, derivatization, and TLC purification requires ~3 hrs; and extraction, drying, and silylation requires ~2 hrs. Although this method is more time intensive when compared to other assays of oxidative stress, quantifying F₃-IsoPs through GC/MS analysis results in an accurate, sensitive, and linear assessment of endogenous oxidative damage.

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Figure 1. Derivatization of 15-series F₃-Isoprostanes for GC/MS analysis

F₃-IsoPs must undergo chemical transformation to the pentafluorobenzyl ester, trimethylsilyl ether derivatives prior to GC/MS analysis. After separation phase extraction/purification, F₃-IsoPs are first derivatized by PFBB, which attacks the carboxyl group of the F₃-IsoP. The pentafluorobenzyl ester moiety of the resulting product is an ideal leaving group for formation of the carboxylate anion detected by the mass spectrometer. Trimethyl silyl groups are added to the hydroxyl groups through derivatization by BSTFA, which functions to lower polarity of the molecule and significantly lowering its boiling point for ease of vaporization when injected into the gas chromatograph. During the ionization process the pentafluorobenzyl ester is cleaved and the carboxylate anion is the resulting species detected by the mass spectrometer.

Figure 2. Representative chromatogram showing the elution of endogenous F_3 -IsoPs in *C. elegans* lysates

A representative selective ion monitoring chromatogram obtained from the analysis of F_3 -IsoPs in *C. elegans* lysates is shown in Fig. 2. The chromatograph peak in the lower m/z 573 ion current chromatogram represents the internal standard d_4 -15- F_{2t} -IsoP. The series of peaks in the upper m/z 567 ion current chromatogram represent putative F_3 -IsoPs eluting over approximately a 1-min interval. These compounds possess a molecular mass predicted for F_3 -IsoPs and elute at the predicted retention time. F_3 -IsoPs are predicted to elute at later

retention times than the deuterated 15-F_{2t}-IsoP internal standard due to F₃-IsoPs containing one more double bond than F₂-IsoPs. The concentration of F₃-IsoPs is calculated using the ratio of the intensity of this peak to that of the internal standard. Levels of F₃-IsoPs in lysates are reported in nanograms per gram of protein content.