Video Article

Prostaglandin Extraction and Analysis in *Caenorhabditis elegans*

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Abstract

Caenorhabditis elegans is emerging as a powerful animal model to study the biology of lipids[1-9]. Prostaglandins are an important class of eicosanoids, which are lipid signals derived from polyunsaturated fatty acids (PUFAs)[10-14]. These signalling molecules are difficult to study because of their low abundance and reactive nature. The characteristic feature of prostaglandins is a cyclopentane ring structure located within the fatty acid backbone. In mammals, prostaglandins can be formed through cyclooxygenase enzyme-dependent and -independent pathways[10,15]. *C. elegans* synthesizes a wide array of prostaglandins independent of cyclooxygenases[6,16,17]. A large class of F-series prostaglandins has been identified, but the study of eicosanoids is at an early stage with ample room for new discoveries. Here we describe a procedure for extracting and analyzing prostaglandins and other eicosanoids. Charged lipids are extracted from mass worm cultures using a liquid-liquid extraction technique and analyzed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The inclusion of deuterated analogs of prostaglandins, such as $PGF_{2\alpha}$-d_4 as an internal standard is recommended for quantitative analysis. Multiple reaction monitoring or MRM can be used to quantify and compare specific prostaglandin types between wild-type and mutant animals. Collision-induced decomposition or MS/MS can be used to obtain information on important structural features. Liquid chromatography mass spectrometry (LC-MS) survey scans of a selected mass range, such as *m/z* 315-360 can be used to evaluate global changes in prostaglandin levels. We provide examples of all three analyses. These methods will provide researchers with a toolset for discovering novel eicosanoids and delineating their metabolic pathways.

Video Link

The video component of this article can be found at https://www.jove.com/video/50447/

Introduction

Prostaglandins (PGs) are an important class of lipid hormones that have been extensively investigated and implicated in regulating reproduction, immunity, and development in a wide array of organisms[12-14]. PGs are ideally suited for comprehensive systems biology analyses because they comprise a series of lipids derived from a common precursor(s). The nematode *C. elegans* is one of the most widely used model organisms to address fundamental questions in genetics and systems biology.

We have demonstrated that *C. elegans* synthesizes F-series PGs that guide motile sperm to oocytes[3,6,16]. F-series PGs derived from 20-carbon PUFAs with three, four, and five double bonds, comprising the F1, F2, and F3 classes, respectively have been identified[3,16]. These PGs are synthesized independent of cyclooxygenase enzymes, yet $PGF_{1\alpha}$ and $PGF_{2\alpha}$ stereoisomers are still generated. For qualitative and quantitative analyses of *C. elegans* PGs, LC-MS/MS is a powerful and sensitive analytical technique. Before performing this analysis, it is important to develop an optimized extraction method because the performance of the analytical process heavily depends on the extract quality. Liquid chromatography and mass spectrometry can only provide anticipated mass to charge ratio (*m/z*), as well as desirable peak shape and retention time of the PG parent ion. Metabolic profiling of PGs in *C. elegans* is a challenging task requiring various MS acquisition strategies.

LC-MS full scan or survey scanning (Q1 scanning) has the advantage of ensuring that most ionizable PGs will generate a mass spectrometric response. While the survey scan provides useful information on total profiling of PGs with respect to wild-type and mutant *C. elegans*, detection of minor PGs is compromised due to low sensitivity. Nevertheless, LC-MS survey scanning can give a global view of PGs and is particularly useful for analyzing mutants predicted to affect metabolism of a large PG population.

In metabolite identification, LC-MS/MS analysis of chemically synthesized PG standards is first performed to obtain their product ion spectra as reference compounds. These spectra can be compared to the spectrum of an unknown metabolite. Multiple reaction monitoring (MRM) mode is used for enhanced sensitivity and specificity. An MRM experiment is accomplished by specifying the parent ion/product ion mass transition of a compound. By knowing the mass and structure of the target analytes, it is possible to predict theoretical MRM transitions for many unknown metabolites.

An optimized LC-MS/MS method for profiling isomeric PGs in *C. elegans* has not been reported. Here we describe an extraction and integrated mass spectrometry approach consisting of LC-MS and LC-MS/MS methods for detecting, quantifying, and investigating *C. elegans* PG metabolites. This approach can be applied to other eicosanoids.

Protocol

The protocol as described below is divided into three sections: worm culture, prostaglandin extraction, and prostaglandin analysis. As noted, there are multiple points when samples can be temporarily stored at -80 °C or -20 °C.

1. Worm Culture

We recommend approximately 6 g of mixed stage worms for comprehensive LC-MS/MS. This should provide enough material for detection at least six separate injections. For quantification of known PGs by MRM in a single injection or two, 1-2 g are sufficient. Analysis of extracts from synchronized cultures consisting of a specific stage is also possible. Up to 4 different strains can be grown simultaneously. For example, a wild type control and three different mutant strains.

1. Prepare 65 X-large (16 cm) NGM plates and concentrated bacteria for supplemental feeding. Use NA22 bacteria for seeding. To make concentrated bacteria for supplemental feeding, grow at least four liters of NA22 in LB medium for 16-24 hr. Spin down, remove the supernatant, and resuspend bacteria in 100 ml of M9 buffer. 200 to 400 ml of this concentrated bacterial solution may be required for each worm strain. Store at 4 °C.
2. Start worm cultures on 5 X-large plates. Grow worms for approximately 3 to 4 generations until plates are full of gravid adults. Concentrated bacteria should be added to plates to prevent starvation, as necessary (~ 1 ml/plate and let dry completely before replacing lid).
3. Wash gravid hermaphrodites off plates with M9 buffer and collect the worms in a 50 ml conical polypropylene tube.
4. Allow gravid hermaphrodites to settle to the bottom (usually about 3-5 min). Remove supernatant, including bacteria, eggs, and larval stage worms. Resuspend in 15 ml M9 buffer and gently mix.
5. Transfer 200 µl of worm suspension to each of 60 seeded NGM plates. Disperse worms across the plates. Keep worm solution mixed well until all plates have been seeded to ensure that they receive roughly equal numbers of worms.
6. Supplement with concentrated bacteria as needed (~1 ml/plate/12 to 24 hr period) to prevent starvation. Allow plates to air dry before replacing lids. Grow worms for 2-4 generations, depending on the original number of seeded worms, or until plates are full of gravid adults.
7. Harvest worm plates one strain at a time. Wash worms off the plates with M9 buffer and collect in 50 ml polypropylene tubes (*e.g.* 6 tubes). Use M9 buffer to wash a plate, then transfer the worm-filled buffer to the next plate. After washing a number (*e.g.* 10th) of plates, transfer the worm-filled buffer to the 50 ml tube. Fill the tube to the top with M9 buffer. Repeat the wash for the rest of the plates.
8. As the gravid adults settle to the bottom in 5-6 min, remove the supernatant (~ 35 ml) and consolidate into one or two tubes. Fill the tubes to 50 ml with fresh M9 buffer, let stand for 3-5 min, and remove supernatant leaving gravid adults. Repeat 3 times or until the supernatant is transparent.
9. Using a large bore Pasteur pipette, transfer as many worms as possible to two 15 ml polypropylene conical tubes. Spin down at 1,000 rcf (~3,500 rpm in CentraSL2) for 5 min in a clinical centrifuge. Remove supernatant and repeat until all worms are transferred and pelleted in the same tube. Remove as much supernatant as possible and store worms at -80 °C. Repeat for all strains.

2. Prostaglandin Extraction

The reagents needed for extraction are shown below. The method is modified from that of Golovko and Murphy[17] and includes acetone extraction followed by liquid-liquid purification, which enhances LC-MS/MS sensitivity. A Bullet Blender 5 homogenizer is used in the procedure, although similar results can be obtained with a Dounce homogenizer. Butylated hydroxytoluene (BHT) and evaporation under Nitrogen (N_2) gas are used to prevent oxidation. All glassware should be siliconized (Sigmacote) to reduce lipid binding. Extraction with organic solvents should be performed in a chemical hood.

1. Weigh a 50 ml polypropylene conical tube. Transfer approximately 6.0 g of frozen worms from the 15 ml conical tube stored at -80 °C by cutting through the tube with a hot razor blade near the 6.5 ml mark. A methanol-cleaned spatula can be used to remove the frozen worm pellet from the bottom of the tube. By cutting through the pellet to retrieve the lower portion, less dense larval worms and residual bacteria from the top of the pellet are left behind.
2. Transfer the frozen worms to the pre-weighed 50 ml conical tube. If multiple samples are being processed, use the same amount (6.0 g) of tissue for comparative studies. As the worms thaw into a paste, add or remove worms with the spatula to obtain the desired mass. Optional: add 1.00 ng of $PGF_{2\alpha}$-d4 standard as an internal control for extraction efficiency.
3. Add 12 ml of ice-cold 2:1 acetone/saline with 0.005% BHT to the worm suspension and mix well by vortexing. Dispense 1.5 ml of the worm slurry to each of twelve 5 ml self-standing plastic tubes for use in the Bullet Blender.
4. Add 0.7-0.8 ml of 0.5 mm diameter Ceria stabilized zirconium oxide beads to each 5 ml tube. Close caps tightly and load the 12 tubes into the Bullet Blender 5 homogenizer. Run the blender for 3 min at speed 8-9 and place the tubes on ice. Be sure to close the caps very tightly or the content may spill out. Check 10 µl of homogenate from one tube on a slide using a stereoscope. There should be very few intact worms remaining. Repeat at the same speed for another minute, if necessary.
5. Evenly transfer the homogenates to four 10 ml conical glass tubes using a 9" Pasteur pipette. Avoid transferring beads. Wash the beads from three tubes sequentially with 1 ml of 1:2 acetone/saline containing BHT. Repeat for other tubes until all beads are washed. Transfer the remaining solution (~ 4 ml) to the 10 ml glass tubes and place them on ice.
6. Centrifuge the 10 ml glass tubes at 4 °C in a clinical centrifuge at ~1,000 x g for 10 min. Transfer the supernatant from each tube to a clean 10 ml glass conical tube.
7. In a chemical hood, add an equal volume of hexane to each 10 ml glass tube. For example, add 3.5 ml hexane to 3.5 ml acetone/saline solution. Vortex at maximum speed for 30 sec.

8. Centrifuge the 10 ml glass tubes at 4 °C in a clinical centrifuge at ~1,000 x g for 10 min. Discard the upper phase containing hexane and neutrally charged lipids.
9. Acidify the lower phase to pH 3.5 using 2 M formic acid (approximately 100 - 150 µl). Test pH using pH strips.
10. Add an equal volume of chloroform to each tube. Vortex at maximum speed for 30 sec and centrifuge the tubes at 4 °C in a clinical centrifuge at ~1,000 x g for 10 min.
11. Remove and discard the upper aqueous phase. Insert a pipette tip through any residual milky interphase to the lower chloroform phase, avoiding the interphase. Collect the lower phase from each of the 4 tubes to a clean 15 ml conical glass tube. Incubate at -20 °C for at least 24 hr. At this point, the extract can be stored at -20 °C for up to two weeks.
12. Take the extract out of the freezer and discard the remaining milky aqueous top layer, if present. Transfer the chloroform phase to a labeled Teflon-lined ½-Dram glass vial using a new Pasteur pipet. In a chemical hood, evaporate the chloroform soluble fraction to dryness under a gentle flow of N_2 gas. The dried extract can be stored at -20 °C for up to 2 weeks. An overall scheme used for PG extraction and analysis is shown in **Figure 1**.

3. Prostaglandin Analysis

1. Prepare stock solutions of individual reference prostaglandins, such as $PGF_{2\alpha}$ or $PGF_{2\alpha}$-d4 (1 µg/ml) in methanol and dilute with methanol:water (8:2 v/v) to obtain a working solution. Prepare a series of dilutions (100, 10, 1, 0.1, 0.01 ng/ml) to generate a standard curve.
2. Add 200 µl methanol:water (8:2 v/v) to the dried *C. elegans* lipid extract(s) and mix thoroughly. If less than 6 g of worm tissue was extracted, the dried extract should be resuspended in less volume of methanol:water solution.
3. Prepare the mobile phase consisting of 0.1% formic acid in water [A] and acetonitrile containing 0.1% formic acid [B].
4. Set up the autosampler at 4 °C and place the sample vials in a 70-count 1.5 ml vial rack.
5. Equilibrate the Synergy hydro RP-C18 column with 0.1% formic acid at a flow rate of 0.2 ml/min for about 5 min.
6. Inject the sample (20-50 µl) into the column. Perform gradient elution starting with 10% B and going up to 80% B from 0-11 min, 80-100% B from 11-14 min, and returning back to 10% B at 16 min. The total run time is 20 min. The retention times of 13 authentic standards are shown in **Table 1** for reference[3].
7. Introduce the column effluent into the mass spectrometer using an ESI interface operating in the negative ion mode. Nitrogen is used as a nebulizer and curtain gas (CUR = 10). The collision gas, collision energy, and temperature are set at 10, -35 eV and 600 °C, respectively. Declustering potential (DP), collision energy (CE), and cell exit potential (CXP) are set at -90, -35 and -10, respectively.
8. For survey scans (Q1 scans), use negative ion mode in the mass range of *m/z* 315-360 for most PGs (**Figure 2**). The range can be altered, depending on the mass of the compound(s) of interest. Extract ions from total ion current (TIC) of LC-MS ion chromatograms and determine whether the extracted ions correspond to deprotonated or adduct ions, or to fragment ions.
9. For MRM experiments, mass transition *m/z* 355/311 is used to detect the F1 class, *m/z* 353/193 is used for the F2 class, and *m/z* 351/193 or 351/191 is used for the F3 class (**Figure 3**). MRM is also used to detect the internal standard (for example, *m/z* 357/197 for $PGF_{2\alpha}$-d4) and to generate the standard curve. See Murphy *et al.* for additional information on PG mass transitions[18].
10. For MS/MS experiments, use *m/z* 355 for F1 class, *m/z* 353 for F2 class, and *m/z* 351 for F3 class PGs. *C. elegans* MS/MS data for F-series PGs is available in **Figure 4** and **Table 2**[3,16]. MS/MS data for mammalian eicosanoids is available at www.lipidmaps.org.
11. Process the analytical data using Analyst software (Version 1.4.2, Applied Biosystems).

Representative Results

Sample preparation was carried out by liquid-liquid extraction adapted from Golovko and Murphy[17]. It provided excellent recovery of the internal standard ($PGF_{2\alpha}$-d4). The general scheme for PG extraction from *C. elegans* is shown in **Figure 1**. Chromatographic conditions were optimized to provide baseline separation of >30 eicosanoid standards (**Table 1** shows 13 standards). A Hydro-Rp column (250 x 2.0 mm i.d) with water and acetonitrile containing 0.1% formic acid provided the best separation and sensitivity. Survey scans of wild-type and *fat-3* mutant extracts shown in **Figure 2** document global levels of ions within the mass range of 315-360 atomic mass units. *fat-3(wa22)* mutants lack most 20-carbon PUFAs. An F3 class PG is highlighted. In **Figure 3**, MRM analyses of a wild-type extract show multiple PG isomers of the F1 (**Figure 3A**), F2 (**Figure 3B**), and F3 (**Figures 3C** and **3D**) classes. The F3 class can be detected with either of two mass transitions, *m/z* 351/193 or *m/z* 351/191. CePGF2 is predominantly comprised of the $PGF_{2\alpha}$ enantiomer. CePGF1 is likely to be $PGF_{1\alpha}$ or its enantiomer. **Figure 4** shows the collision-induced decomposition of CePGF2 (RT=11.8) compared to the $PGF_{2\alpha}$ standard (RT=11.8). Minor differences in product ions are likely due to low CePGF2 abundance and co-eluting compounds in the extract.
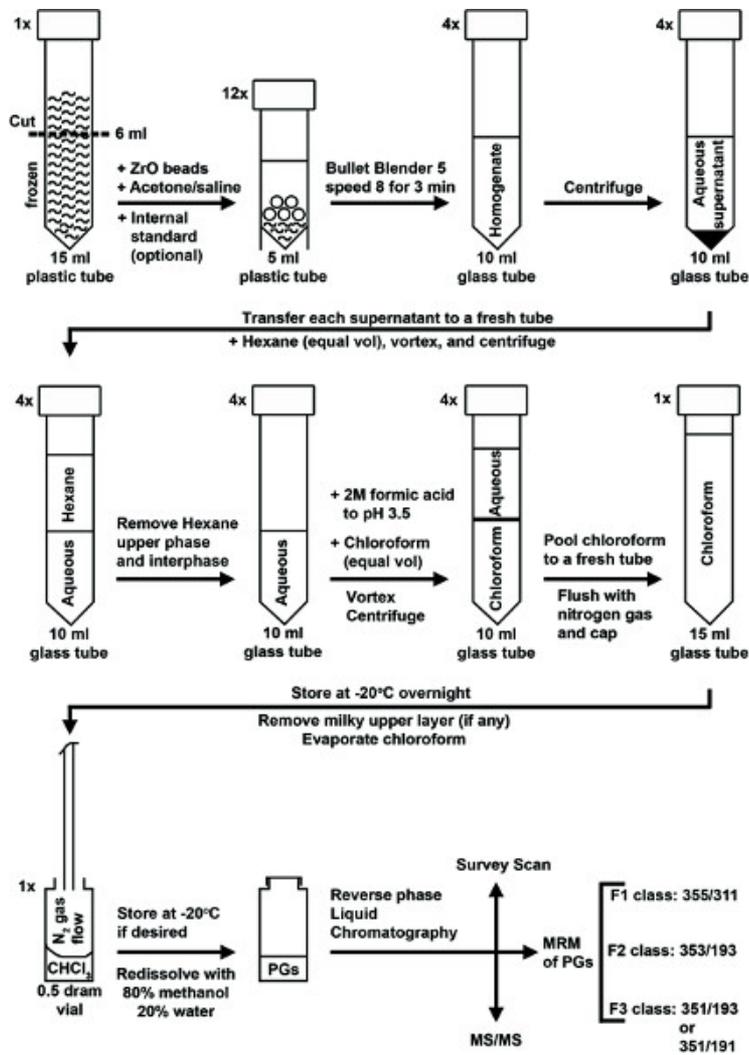
Figure 1. Schematic diagram of *C. elegans* PGs extraction and LC-MS analyses. Note that acetone/saline and chloroform both have 0.005% BHT to minimize lipid oxidation. Refer to the text for more details. Click here to view larger figure.
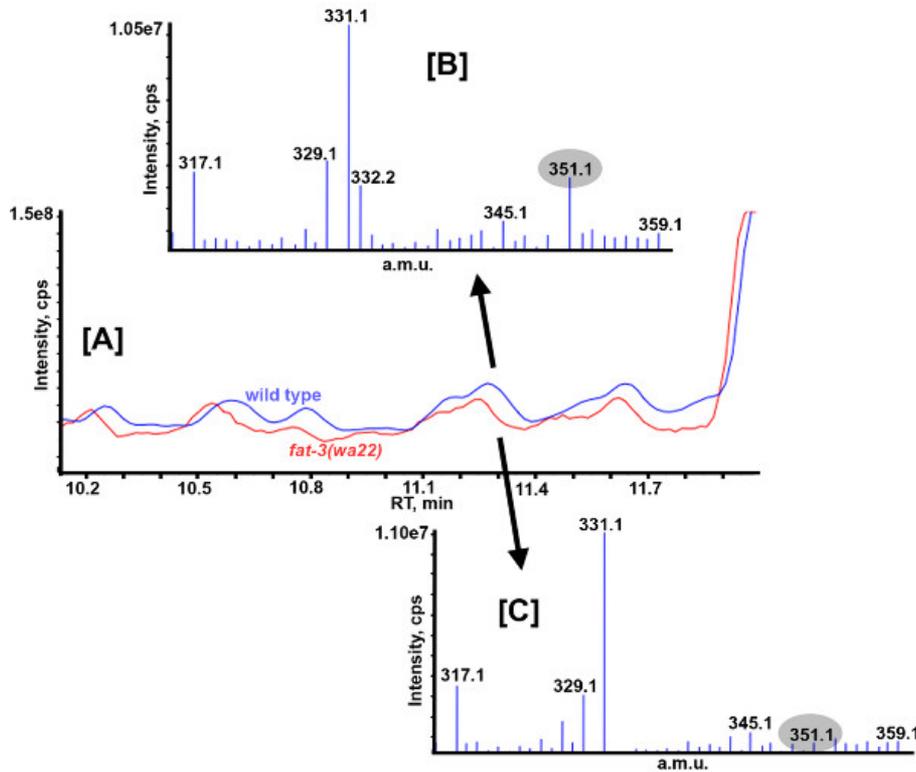
Figure 2. Survey scans of *m/z* 315-360 of wild-type and *fat-3(wa22)* mutant extracts. Liquid chromatography retention time (RT) is shown in panel **[A]**. Extracted ions at RT = 11.3 are shown for wild-type **[B]** and *fat-3(wa22)* **[C]** extracts. An F3 class PG with mass *m/z* 351 is highlighted. Cps, counts/sec; a.m.u., atomic mass unit.
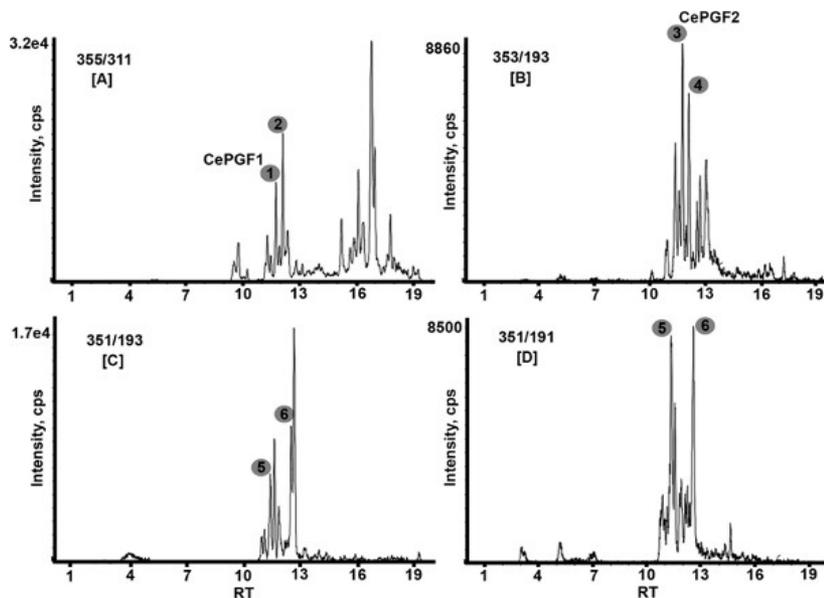

Figure 3. MRM analyses of wild-type extracts. F1 class PGs are detected with mass transition *m/z* 355/311 **[A]**. Notice that several hydrophobic compounds (RT > 14 min), which are unlikely to be PGs, are also detected with this transition. F2 class PGs are detected with mass transition *m/z* 353/193 **[B]**. F3 class PGs are detected with either mass transition *m/z* 351/193 **[C]** or *m/z* 351/191 **[D]**. Notice that the extracts contain multiple PG isomers of each class. Numbers correspond to PGs shown in **Table 2**. The concentration of CePGF2 is 1.8 ng/ml. RT, retention time; cps, counts/sec.
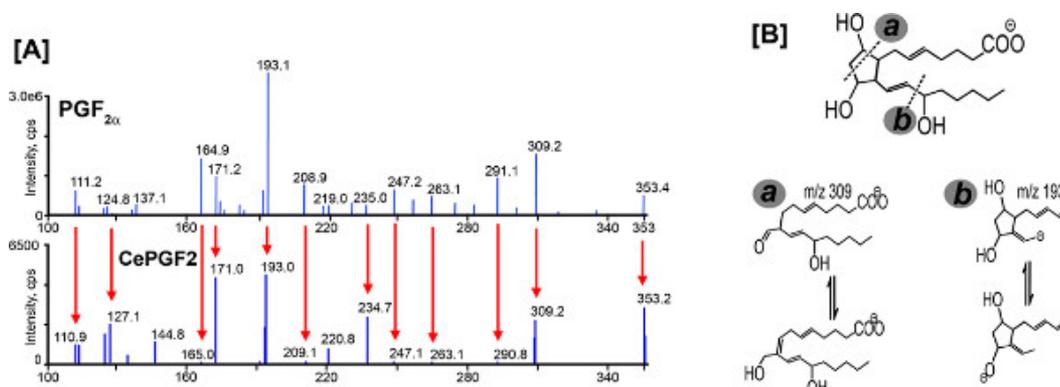
Figure 4. Collision-induced decomposition of chemically synthesized PGF$_{2\alpha}$ and CePGF2. Arrows in panel **[A]** indicate product or fragmentation ions shared between PGF$_{2\alpha}$ and CePGF2 (RT=11.8). The product ions at *m/z* 309 and *m/z* 193 are generated from indicated cleavage sites (highlighted *a* and *b*), correspond to the structures shown, and are characteristic of F-series PGs **[B]**. Cps, counts/sec. Click here to view larger figure.

Standard	RT (min)	[M-H]- *m/z*	Key product ions in MS/MS
20-hydroxy PGE$_2$	9.43	367	349, 331, 287, 234, 189, 129, 109
PGF$_{3-}$	11.26	351	333, 307, 289, 271, 245, 219, 209, 193, 191, 171, 165, 111
Thromboxane B$_2$	11.66	369	289, 191, 177, 169, 151
PGF$_{2-}$	11.80	353	335, 309, 291, 273, 263, 247, 235, 209, 193, 171, 165, 111
PGF$_{1-}$	11.79	355	337, 319,311, 301, 293, 275, 265, 237, 211, 195
Lipoxin B$_4$	12.38	351	201, 191,189, 165, 155, 115, 107, 71, 59
PGD$_2$	12.56	351	315, 271,203, 189
5(S), 6(R)- Lipoxin A$_4$	12.83	351	235, 217, 189, 144, 135, 115, 99, 59
PGA$_2$	14.02	333	315, 297, 271, 235, 191, 189, 175, 163, 137, 113, 109
Δ12-PGJ$_2$	14.20	333	271, 189, 123
Leukotriene B$_4$	14.73	335	181, 109, 93, 71, 69, 59, 57
15d-Δ^{12,14}-PGJ$_2$	16.80	315	297, 271, 217, 203, 158
5(S)-HpETE	17.88	335	97, 83, 81, 57

Table 1. Retention times and key product ions of 13 eicosanoid standards from the LC-MS/MS method[3] . Over 30 standards were used to develop the LC-MS/MS program. The chromatographic retention times (RTs) differ, even among very similar PGs. An exception is PGF$_{1\alpha}$ and PGF$_{2\alpha}$. Nevertheless, these PGs are distinguished by their parent ion masses ([M-H]- *m/z*) and collision-induced decomposition spectra (MS/MS).

Prostaglandin class	No. in Figure 3	[M-H]- m/z	Key product ions in MS/MS
F1 (CePGF1)	1	355	319, 301, 311, 293, 275, 265, 237, 223, 211, 195, 157
F1	2	355	311, 301, 293, 275, 265, 211, 195, 167, 157
F2 (CePGF2)	3	353	309, 291, 273, 263, 247, 209, 193, 171, 165, 127
F2	4	353	309, 291, 273, 263, 255, 247, 219, 209, 193, 171, 113
F3	5	351	315, 307, 289, 275, 249, 205, 193, 191, 167, 153, 139
F3	6	351	333, 289, 271, 261, 245, 223, 193, 191, 163

Table 2. Collision-induced decomposition product ions for several major *C. elegans* F1, F2, and F3 PGs shown in Figure 3. MS/MS of other less abundant isomers are not shown. These data are from multiple analyses and not all product ions may be visible in a given run. Given the complexity of the extracts, some less abundant product ions may derive from another parent ion with similar retention time or result from interference. Peak 2 is likely a stereoisomer of CePGF1 and peak 4 is likely a stereoisomer of CePGF2. See Edmonds, *et al.* (2010) for additional data[3].

Discussion

We describe a procedure for eicosanoid extraction and analysis, focusing on F-series PGs. There are several parts of the procedure that can be problematic. First, it is critical that the worm cultures do not starve, as starvation can alter PG metabolism. For supplemental feeding, NA22 bacteria are recommended instead of the more commonly used OP50 bacteria because NA22 reaches higher density. However, the NA22 strain lacks antibiotic resistance and is more susceptible to contamination. Second, worms synthesize individual PG isomers in low abundance relative to mammalian tissues. This may be due in part to redundancy among the numerous isomers. We recommend about 6 g of tissue for comprehensive analysis and stronger signals. Less tissue (1-2 g) can be used if the dried extract is resuspended in less methanol:water solution to increase PG concentration. However, only one to two injections can be performed. The detection limit of our LC-MS/MS system is about 10 pg $PGF_{2\alpha}$/ml. One ml of densely packed mixed stage worms (about 1 g) yields roughly 25-50 pg of CePGF2. More sensitive mass spectrometry systems can reduce the amount of worm tissue required for analysis. Third, differences in PG extraction efficiency among samples can cause variable results. We have found that extraction efficiency is very similar when tissues are extracted in parallel. To determine the efficiency, add 1.0 ng of $PGF_{2\alpha}$-d4 at the homogenization step as an internal control. MRM using mass transition *m/z* 357/197 is used to measure $PGF_{2\alpha}$-d4 concentration relative to a 1 ng/ml standard solution. The amount of $PGF_{2\alpha}$-d4 lost during extraction is then calculated.

The chromatography parameters and mass transitions we include can be altered to detect other PGs and eicosanoids. Despite considerable effort, we have not been able to identify D-series or E-series PGs in the extracts[17]. Furthermore, we have not detected 8-iso $PGF_{2\alpha}$ and 8-iso PGE_2 that are characteristic of free radical-initiated peroxidation, which generates a nonselective mixture of PG stereoisomers[19]. Whether worms synthesize other PG types is not known. Endocannabinoids and various epoxy and hydroxy metabolites of arachidonic and eicosapentaenoic acids have been found in *C. elegans* extracts[5,7,20,21]. We recommend the use of both MRM and MS/MS compared to authentic standards for quantification and identification, respectively. For novel eicosanoids, these analyses should be combined with a comparative study of *fat* mutants, which are deficient in PUFA synthesizing enzymes[22], as well as a functional assay, if possible. A caveat to analyzing *fat* mutants is that minute quantities of PUFAs present in worms are sufficient for PG synthesis. For example, *fat-2(wa17)* mutants have a small amount of D12 desaturase activity (~5% of wild type[22]) and these mutants still produce PGs[6]. *fat-3(wa22)* and *fat-4(wa14)* mutants fail to synthesize PGs derived from arachidonic and eicosapentaenoic acids[16]. We also have found that *fat* mutants compensate for the loss of PUFA classes by up-regulating PG synthesis from remaining classes. For example, *fat-3(wa22)* mutants fail to synthesize most PGs derived from 20-carbon PUFAs. Instead, they appear to up-regulate novel PGs derived from 18-carbon PUFAs[16]. To date, we have not identified a *C. elegans* strain that is completely deficient in PG synthesis. It is possible that PGs are essential for growth or development.

Disclosures

The authors have no conflicts of interest.

Acknowledgements

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