

Identification of unresolved complex mixtures (UCMs) of hydrocarbons in commercial fish oil supplements

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Abstract

BACKGROUND: Heightened awareness of the health benefits of fish oil consumption has led to a great increase in the number of fish oil supplements available to the consumer. Therefore manufacturers are continually looking for ways to distinguish their products from those of competitors. Minimally refined or virgin fish oils provide a unique feature; however, petroleum hydrocarbon contamination from oil spills is a reality in the world's oceans. The question arises whether oil produced from fish species caught in these polluted areas is free of petroleum hydrocarbons, with particular interest in unresolved complex mixtures (UCMs). This study investigates the presence of UCMs in commercially available fish oil supplements advertised as being virgin, as well as refined.

RESULTS: Weathered petroleum hydrocarbons in the form of a UCM were found at $523 \mu\text{g g}^{-1}$ in a virgin Alaskan salmon oil supplement. Supplements that were refined were free of this contamination.

CONCLUSION: Fish used in the production of fish oil supplements appear to have accumulated petrogenic hydrocarbons in their tissues which were not removed by minimal oil refining. Further study is required to determine if there are any health implications associated with long-term consumption of these contaminated supplements.

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Keywords: unresolved complex mixture; petrogenic hydrocarbons; salmon oil; dietary supplements; fish oil refining

INTRODUCTION

Hydrocarbon contamination is an unfortunate reality in the world's oceans. Contamination can be due to oil spills, e.g. the notable Exxon Valdez in Prince William Sound, Alaska (1989) and Deepwater Horizon in the Gulf of Mexico (2010), or to offshore drilling and land run-off.¹ Evidence of oil spills remains even decades after a spill has occurred.² Petroleum hydrocarbons are normally analyzed by gas chromatography (GC); however, over time, the chromatographic resolution of these hydrocarbons decreases as weathering and microbial degradation modify the original structures, until resolution is so poor that only a broad peak of very similar and unresolved structures remains.² These are known as unresolved complex mixtures (UCMs). UCMs are particularly resistant to biodegradation and persist in the environment for many years.³

UCMs differ in composition depending on a number of factors, including age and source of contamination. Although their name implies a great deal of uncertainty surrounding the compounds within them, the use of techniques such as two-dimensional GC/GC and two-dimensional gas chromatography/time-of-flight mass-spectrometry (GC/GC/TOF-MS) has allowed the identification of many compounds contained in UCMs.^{4,5} UCMs may contain a mixture of both aliphatic and aromatic hydrocarbons, as well as more polar compounds such as carboxylic acids or sulfur-containing compounds.^{5–8} Fractionation techniques can be used to separate each of these groups for more detailed analysis.

A large body of research has reported the toxic effects of aromatic UCM hydrocarbons in the marine mussel *Mytilus edulis* (Linnaeus, 1758).^{5,9,10} Donkin *et al.*⁹ demonstrated that mussels living in contaminated areas bioaccumulated hydrocarbons, including UCM hydrocarbons, and experienced decreased feeding rates. In that study the toxicity experienced by mussels living in contaminated areas was predominantly due to the aromatic hydrocarbons within the UCM.⁹ Aliphatic UCM hydrocarbons are generally given less attention as they have been found to be relatively non-toxic to mussels.^{9,11} However, Thomas *et al.*¹¹ showed that compounds resulting from the oxidation of aliphatic hydrocarbons within a UCM had toxic effects on mussels. Adverse effects due to petroleum UCMs are not uncommon. For instance, Scarlett *et al.*¹² found that growth rates and reproductive success were reduced in the amphipod *Corophium volutator* (Pallas) when chronically exposed to sediment containing a UCM.

Fish can also accumulate hydrocarbons from the environment through their gills and from their diet.^{13–15} Once taken in by the gills, hydrocarbons circulate through the body of the fish via the

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circulatory system, where they can be deposited in muscle and adipocytes or stored in the liver or gall bladder.^{13,16} Unlike invertebrates, fish have livers that contain aryl hydrocarbon hydroxylase enzyme which metabolizes hydrocarbons.^{17,18} Less research has been conducted on the absorption and accumulation of aliphatic hydrocarbons; the studies pointing to lowered toxicity of aliphatic hydrocarbons in mussels seem to have been extrapolated to fish. However, aliphatic hydrocarbons have been found to accumulate in the tissue of several fish species.¹³ Mironov *et al.*¹³ found the accumulation of biogenic and branched hydrocarbons (some of which suggest petroleum contamination), as well as a UCM indicative of petrogenic contamination, in several species of fish caught in the Straits of Malacca and the Mediterranean Sea.

The toxicity and potential for bioaccumulation of weathered petroleum hydrocarbons raise concerns over the transfer of these pollutants to humans through the consumption of marine species and increasingly popular fish oil supplements. This issue is becoming more relevant as consumers become aware of the numerous health benefits associated with long-chain omega-3 fatty acids present in marine species and as manufacturers are advertising the 'purity' and benefits of minimally refined fish oil supplements. Typically, fish oils used in dietary supplements undergo a number of refining processes such as molecular distillation and steam deodorization to remove oxidation products, contaminants, including hydrocarbons, and other non-triglyceride compounds.¹⁹ Molecular distillation involves heating a thin layer of oil under vacuum and cooling it rapidly to remove free cholesterol, hydrocarbons, polychlorinated biphenyls (PCBs), oxidation products and free fatty acids.²⁰ Steam deodorization is similar to molecular distillation, except that steam is used to strip volatile compounds that negatively affect sensory perception of the oil. Both these processes require high temperatures and can reduce concentrations of, or completely remove, naturally occurring antioxidants such as carotenoids and tocopherols. Therefore a number of manufacturers prefer to use milder refining techniques to ensure that these antioxidants, and associated color, remain in the oil. Virgin oil supplements generally bypass purification steps that involve elevated temperatures, such as molecular distillation and steam deodorization, to produce minimally refined oil which is then encapsulated.

The objective of this study was to determine if weathered petroleum hydrocarbons, in the form of a hydrocarbon UCM, were present in minimally refined fish oil supplements sold in Canada and the USA.

EXPERIMENTAL

Three commercial fish oil supplements were purchased at a local grocery store for analysis. Two of the supplements (referred to as brands A and B) were capsules labeled as wild salmon oil and were selected based on claims of being 'virgin' or minimally refined. Supplement brand A's capsule shell was labeled as containing gelatin, glycerin and water, while supplement brand B was encapsulated in an enteric-coated shell consisting of gelatin, glycerin, water, ethyl cellulose, medium-chain triglycerides, oleic acid, sodium alginate and stearic acid. A third supplement (referred to as brand C) was a liquid sardine, anchovy and mackerel blend that was molecularly distilled to remove impurities. Since two of these supplements were stated to be salmon oil of Alaskan/Pacific origin, they were compared with an Alaskan salmon oil that had undergone molecular distillation to refine the oil and remove impurities (referred to as refined salmon oil). All chemicals and

solvents (Optima grade) were purchased from Fisher Scientific Company (Guelph, ON, Canada) unless stated otherwise.

Zhou *et al.*'s²¹ method for extracting aliphatic and aromatic hydrocarbons was followed, with some modifications. Approximately 0.2 g of the capsule contents or liquid fish oil was saponified by refluxing for 1 h with 2 mol L⁻¹ KOH_(aq) with pentacosane (C₂₅) (Sigma-Aldrich, Oakville, ON, Canada) added as an internal standard. The mixture was then transferred to a separatory funnel and washed with 130 g kg⁻¹ NaCl solution. Dichloromethane was used to recover the unsaponifiables. These were concentrated by evaporation to near dryness in a rotary evaporator to minimize loss of volatile short-chain hydrocarbons. The dichloromethane was replaced with hexane by adding 5 mL of hexane to the concentrated unsaponifiables and evaporating to 1 mL under a gentle stream of nitrogen. This was repeated two times. After the final addition of hexane, the unsaponifiables were concentrated to 0.8 mL and added to a silica Sep-Pak (Waters, Milford, MA, USA) to remove more polar compounds. The hydrocarbons were recovered by elution with approximately 6 mL of hexane. The eluate was concentrated to 0.8 mL under a gentle stream of nitrogen, and this process was repeated using a second Sep-Pak.

Zhou *et al.*²² validated this extraction method by carrying aliphatic (*n*-heneicosane and *n*-pentacosane) and aromatic (including naphthalene, fluorene, anthracene, pyrene, benzopyrene and others) hydrocarbons through the procedure. All had recoveries ranging from 86 to 107%, demonstrating that this method isolated both classes of hydrocarbons.

Hydrocarbon analysis was performed on a Bruker 430-GC with an SAC-5 column (poly(5% diphenyl/95% dimethyl siloxane), 30 m × 0.25 mm i.d.; Supelco, Bellefonte, PA, USA) equipped with a flame ionization detector (FID). Splitless injection was used with an injector temperature of 250 °C. The following temperature program was applied: initial temperature of 60 °C held for 15 min, then ramped to 280 °C at 13 °C min⁻¹ and held for 5 min and subsequently ramped to 300 °C at 50 °C min⁻¹ and held for 10 min. The FID was set to 300 °C. Samples were analyzed in triplicate from a single bottle of each supplement. To investigate the structures of the hydrocarbons and confirm the identities of flavoring components, samples were also analyzed by GC/MS (Trace GC Ultra with Polaris Q, Thermo Scientific, Waltham, MA, USA) in electron ionization mode. Hydrocarbons were separated on a free fatty acid phase (FFAP; nitroterephthalic acid-modified polyethylene glycol) column (30 m × 0.25 mm i.d.; Phenomenex, Torrance, CA, USA). The oven temperature was held initially at 60 °C for 15 min, then ramped to 250 °C at 13 °C min⁻¹ and held for 30 min. The identities of well-resolved peaks of pristane, squalene and limonene were confirmed by spectral matches with the National Institute of Standards and Technology (NIST) mass spectral library. Saturated straight-chain hydrocarbons were identified by comparison of both retention times with authentic standards and mass spectra with the NIST library. To explore the UCM, extracted ion chromatograms were used because aliphatic and aromatic hydrocarbons coeluted from the silica Sep-Pak with the extraction procedure used here, and previous studies have shown that aromatic components of UCMs can be toxic to organisms.^{5,10} Specifically, base peak ions indicative of alkylbenzenes (91 and 105), alkylindenes (129 and 143) and alkylnaphthalenes (141 and 155)^{5,23} were targeted, as well as ions representative of the original 16 priority polycyclic aromatic hydrocarbons (PAHs) identified by the US Environmental Protection Agency.²⁴

Using C₂₅ as an internal standard and the mass of oil analyzed, individual peak areas of pristane and squalene and the combined

Table 1. Total hydrocarbon (HC), pristane, squalene and UCM concentrations in three commercial fish oil supplements and refined salmon oil

Supplement brand	Level of refining	Fish species	Fish origin	HC ^a ($\mu\text{g g}^{-1}$)	Pristane ($\mu\text{g g}^{-1}$)	Squalene ($\mu\text{g g}^{-1}$)	UCM ($\mu\text{g g}^{-1}$)
A	None	Salmon	Alaska	548 \pm 80	19.8 \pm 2.8	3.0 \pm 0.9	523 \pm 90
B	Minimal	Salmon	Northern Pacific	40.7 \pm 8.2	10.1 \pm 1.7	2.5 \pm 0.4	ND
C	Full	Sardine, anchovy, mackerel	Peru	20.0 \pm 4.5	0.009 \pm 0.004	3.8 \pm 1.7	ND
Refined salmon oil	Full	Salmon	Alaska	11.9 \pm 3.5	0.016 \pm 0.007	4.1 \pm 0.6	ND

Values are mean \pm standard deviation. ND, not detected.
^a Hydrocarbons summed include both petrogenic and biogenic sources.

peak areas of the UCM were converted to concentrations to quantify these compounds. Similarly, total hydrocarbon concentration was determined by summing peak areas from 16 to 40 min.

RESULTS

All supplements contained biogenic hydrocarbons, including pristane and squalene, at a range of concentrations (Table 1). Brand C, the refined sardine, anchovy and mackerel oil supplement, contained prominent peaks of limonene isomers (Fig. 1D). These are flavoring components commonly used in liquid fish oils, as well as some capsules, to mask odors and flavors that may

develop over time owing to oxidation. Since they are not biogenic or petrogenic hydrocarbons, limonene isomers were not included in the calculation of total hydrocarbons. Similarly, the traces of sterols identified in supplement brand C and the refined salmon oil were not included in the total hydrocarbon concentration. The presence of sterols was expected as they were declared on the nutritional panels of these supplements.

Supplement brand A, marketed as being virgin oil, contained an obvious UCM (Fig. 1A). GC/MS extracted ion chromatograms of the UCM did not reveal any evidence to indicate the presence of aromatic hydrocarbons. Mass spectra were instead consistent with aliphatic cyclic or unsaturated hydrocarbons, with base peak ions

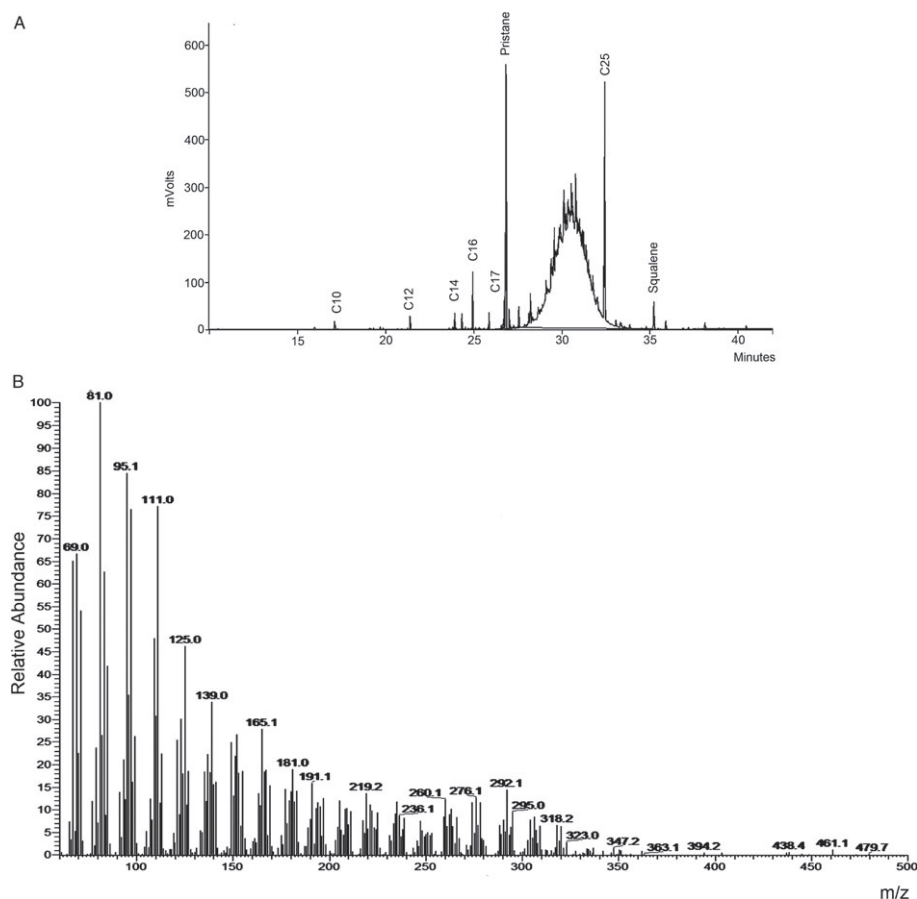


Figure 1. Hydrocarbon fractions recovered from fish oil supplements: A, chromatogram of brand A; B, mass spectrum of UCM in brand A; C, chromatogram of brand B; D, chromatogram of brand C; E, chromatogram of refined salmon oil. The UCM appears between pristane and the internal standard pentacosane (C_{25}) in A.

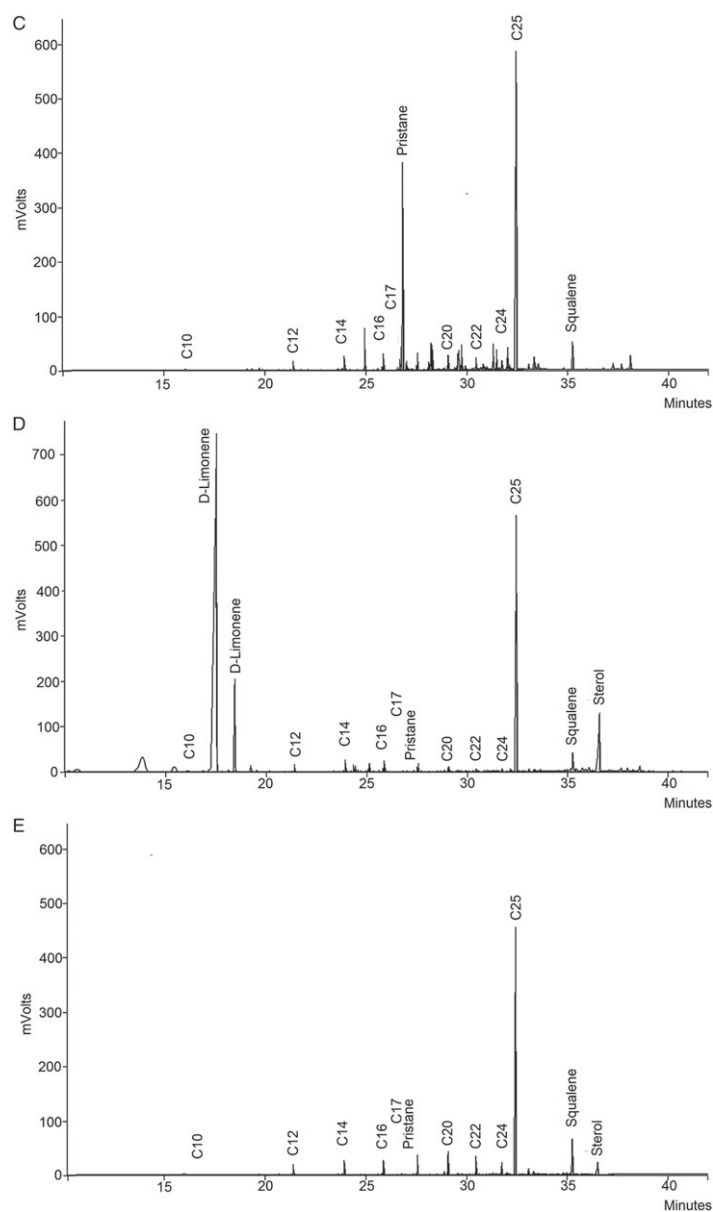


Figure 1. Continued.

of 67, 81 and 95 (Fig. 1B) and showing a clear decrease in abundance with increasing *m/z* ratio. Molecular ions were not obvious. All other supplements contained well-resolved straight-chain saturated hydrocarbons (Figs 1C and 1D).

The UCM of aliphatic hydrocarbons in supplement brand A suggested that weathered petroleum hydrocarbons had bioaccumulated in the fish tissue from which the oil was recovered.²⁵ To exclude the capsule shell as the source of the UCM found in supplement brand A, the shell was also analyzed for hydrocarbon content. The shell was found to contain a UCM but only at trace levels. Contamination of the inner capsule material with oil was the likely source of the UCM in supplement brand A's capsule shell. Supplement brand B, the other wild salmon oil supplement analyzed, contained the second greatest amount of total hydrocarbons at 41 µg g⁻¹ sample without displaying a UCM; its most prominent aliphatic hydrocarbon was pristane at 10 µg g⁻¹ sample. A large quantity of pristane, 20 µg g⁻¹ sample, was also found in supplement brand A (Table 1). Supplement brand C (refined fish

oil supplement) and the refined salmon oil contained the lowest amount of hydrocarbons, with the refined salmon oil containing the least (Table 1). Pristane was present at a level of less than 0.1% of total hydrocarbons in supplement brand C and at a level slightly greater than 0.1% in the refined salmon oil.

DISCUSSION

Hydrocarbons found in aquatic sediment and marine species can be derived from natural sources through biological processes (biogenic) or from anthropogenic sources such as petroleum contamination (petrogenic).²⁶ Biogenic hydrocarbons are found in all living beings, so a distinction between biogenic, generally considered harmless, and petrogenic, more toxic, hydrocarbons must be made.²⁷ A significant number of the defined alkanes present in the gas chromatograms shown here (Figs 1C–1E), particularly C₁₅–C₃₅, are biogenic.²⁸ The distribution of these naturally occurring hydrocarbons generally shows a predominance of odd

carbon-numbered *n*-alkanes, particularly C₁₅ and C₁₇; in contrast, there is no predominance of even or odd number chain lengths in petroleum hydrocarbon distributions.²⁵ However, the presence of a UCM of hydrocarbons in a gas chromatogram is a distinguishing element of weathered petroleum residue.²⁵

Supplement brands A and B both contained pristane at levels of 10 µg g⁻¹ or greater. These supplements were marketed as being minimally refined. Pristane and phytane are present in most petroleum products and therefore their presence may indicate petroleum contamination;²⁵ however, they are also synthesized by algae and could therefore accumulate in fish tissues through diet.^{25,29} Thus the source of pristane in these supplements cannot be determined; however, further refining likely reduces the concentration of this hydrocarbon, as very little was noted in the refined salmon oil and supplement brand C.

The product literature for brand B indicated that the oil was exposed to 'less processing' than other fish oils. Since this product was derived from wild salmon harvested in the North Pacific, we had anticipated that it would contain a prominent hydrocarbon UCM, similar to supplement brand A. However, 'less processing' was not defined and it is impossible to know the extent of the refining process. The very low pristane concentration (<0.02 µg g⁻¹) in brand C and the refined salmon oil suggests that pristane is effectively removed during refining. Higher levels of 10 µg g⁻¹ in brand B, however, suggest that this oil did not experience the same extent of refining. The pristane concentration in brand B is also similar to that in brand A at 20 µg g⁻¹. Thus the lack of a UCM in supplement brand B may simply be because weathered petrogenic hydrocarbons were not present in the salmon tissue from which the oil was recovered. In fact, the manufacturer claimed that the location of the salmon harvest had not been affected by oil spills. It may be that minimal refinement of fish oils is appropriate when the quality of the source material can be guaranteed. Screening of fish tissues for hydrocarbon contamination before processing would be prudent. Extra purification steps may be necessary to remove petrogenic hydrocarbons if a UCM of hydrocarbons is found in supplements containing unrefined oil (Fig. 1A).

The presence of UCMs in salmon oil supplements being sold within Canada and the USA is a concern, because there is evidence that sublethal exposure to UCMs affects the reproduction and growth rate of marine species.¹² Scarlett *et al.*¹² found that sediment containing approximately 435 µg g⁻¹ UCM had a significant negative effect on the growth rate and reproduction of juvenile *C. volutator*. Amphipods associate with hydrocarbons in the sediment through feeding and therefore were unlikely to consume the entire amount of UCM to which they were exposed.¹² Although it is difficult to compare UCM concentrations in sediments and oils, the levels found in the sediments are similar to those found in the unrefined salmon oil supplement, brand A (Table 1). A consumer taking the recommended 2 g daily serving of brand A would be consuming approximately 1 mg of UCM hydrocarbons. A direct relationship between the effects of chronic UCM exposure in *C. volutator* and humans cannot be made, but it is concerning that biological effects have been noted in marine species. The Canadian Council of Ministers of the Environment (CCME, http://www.ccme.ca/assets/pdf/phc_standard_1.0_e.pdf) has set tolerable exposure levels for aliphatic and aromatic petroleum hydrocarbons found in soil; however, human exposure to UCMs in foods has not been addressed.

In Canada, there are no regulations regarding the maximum level of petroleum hydrocarbons allowable in fish oil to be used for dietary supplements. For contaminants, Health Canada

(<http://webprod.hc-sc.gc.ca/nhp/bdipsn/monoReq.do?id=88&lang=eng>) has only set limits on the levels of dioxins, dioxin-like PCBs and PCBs. In light of the findings of this study, it is strongly recommended that further research be conducted on the safety of UCMs from weathered petroleum hydrocarbons for long- and short-term human consumption, since the literature has documented their negative impact on marine life. Depending on the findings, it may be necessary to establish limits and testing requirements for supplements being sold to consumers to ensure that they are safe for consumption.

CONCLUSION

This work has suggested that petroleum hydrocarbons are accumulating in salmon lipids and that, without further refining, these weathered hydrocarbons remain in supplements made from fish oil. Alaskan salmon oil that was refined (molecularly distilled) contained very few hydrocarbons and no UCM, suggesting that the refining process is sufficient to remove weathered petroleum hydrocarbons. This study is the first to our knowledge to demonstrate that UCMs of weathered petroleum hydrocarbons bioaccumulate in fish lipids, whence they can be transferred to humans. With the increase in popularity of fish oil supplements and thus increased consumption of these products, our data suggest that chronic exposure to petrogenic hydrocarbons could be occurring in relatively large numbers of people. Further studies using more advanced analytical techniques such as GC/GC/MS that may permit identification of toxic components within the fish oil, or toxicity studies on the fish oil extracts, will need to be carried out to establish risks associated with regular consumption of unrefined fish oil.

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