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Determination of linear alkylbenzenes in fish tissue by gel permeation chromatography and gas chromatography–mass spectrometry☆

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Abstract

A gel permeation chromatographic (GPC) procedure is described for separation of the long-chain linear alkylbenzenes (LABs) from fish muscle tissues. This technique was found to remove the matrix interference significantly and thereby improve the sensitivity of detection of LABs in the extracts. Gas chromatography–mass spectrometry (GC–MS) was then used to quantitate LABs in different species of fish and also fish from different locations on a transect from the outfalls from the Orange County Sanitation Districts, in the coastal waters of Southern California. The results are consistent with the proximity of the (sewage) source. The data, thus, show that LAB contents in fish tissues, especially that from white croaker (*Genyonemus lineatus*) are useful in assessing exposure to sewage residues in the marine waters. This method of concentrating LABs by GPC could be applicable to all other similar biological matrices.

Keywords: Fish; Alkylbenzenes

1. Introduction

Several studies have reported the presence of long-chain linear alkylbenzenes (LABs) in marine sediments adjacent to urban centers and linked their sources to municipal wastes (i.e. Refs. [1–4]). From the occurrence of LABs in the pelagic fish, Albaiges et al. [5] pointed out earlier their persistence and wide distribution in the marine environment. LABs have since been detected in bivalves and invertebrates [6–9]. LABs have also been found in tissues of freshwater fish from the Fox River System, WI, USA [10]. However, they have not been exploited adequately as molecular markers of sewage contamination in marine organisms such as fish probably, because of an earlier study by Werner and Kimerle [11] that concluded that LABs could be metabolized by fish. Further, matrix interference from the large biomolecules in fauna is a serious hindrance to accurate quantitation of LABs in the lipid fraction. Gel permeation chromatography (GPC) clean-up has been an essential sample preparation technique of lipophilic compounds such as chlorinated pesticides and polychlorinated biphenyls (PCBs) [12–14]. We

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now report a separation technique to concentrate LABs from fish tissue extracts using GPC. The quantitation of LAB homologs is then performed by gas chromatographic–mass spectrometric analysis analogous to that from sediments. The physicochemical properties of LABs are akin to those of many hydrophobic organic and toxic pollutants such as PCBs and polycyclic aromatic hydrocarbons (PAHs). Therefore, LABs could potentially serve as proxies for the fate and transport of other hydrophobic contaminants as well, although they themselves are not known to be environmentally hazardous. Fish muscle tissues were chosen for this study since they reflect the effects of chronic contaminant exposures in the environment [5]. The technique reported here should help widely exploit the utility of LABs in fish as sewage indicators in coastal environments. In general, the separation of the LABs fraction by GPC demonstrated here could be applicable to all other similar biological matrices (i.e. aquatic fauna) around the globe.

2. Experimental

2.1. Reagents

A standard mixture for calibrating the gel permeation chromatographic column was prepared as follows: known amounts of corn oil, di-*n*-octyl phthalate (from Sigma) and 2,4,6-trichlorophenol (Kodak) were dissolved in 100 ml of dichloromethane. A concentrated mixture of linear alkylbenzenes (C_{11}–C_{14} homologs) from Proctor and Gamble was used to make up the working standard solution. All solvents were glass-distilled and the chemicals were of analytical reagent grade. Dichloromethane was used as the mobile phase for GPC.

2.2. Instrumentation

A Fisher 25 mm×1000 mm glass column equipped with an on-column injector valve was packed with Bio-Rad SX-3 Bio-Beads. An FMI medium-pressure pump with a stainless steel head and ceramic piston was used for solvent delivery. The pump was equipped with a Swagelok adjustable pressure relief valve set to ~100 p.s.i. (1 p.s.i.= 6894.76 Pa). Compound elution was followed with a Dionex UV–Vis detector using a 254 nm filter.

A Virtis Tissumizer was used to homogenize and solvent-extract the fish samples. The sample extracts were filtered with Whatman GF/D glass fiber filters (25 mm, disposable) before GPC.

Gas chromatographic–mass spectrometric analysis of the fractions were carried out with a Finnigan 4000 quadrupole mass spectrometer interfaced with a Varian 9610 gas chromatograph equipped with a DB-5 fused-silica capillary column from J&W (30 m×0.25 mm I.D., 0.25 μm phase film). The GC was equipped with a Grob type injector (splitless) with a 2.7 mm I.D. straight bore quartz liner. The linear velocity of the carrier gas, helium, was 35 cm/s at 75°C. The GC column was held at 35°C for 6 min and ramped to 280°C at 4°C/min and then to 310°C at 2°C/min. The MS operating conditions were: EM voltage 1400 V; electron energy 70 eV; source temperature 240°C; injector at 290°C and detector at 300°C. An INCOS Model 2300 data system was used to collect data.

2.3. Sample preparation

Three different fish species, chub mackerel, California scorpionfish, and Pacific sanddab (*Scomber japonicus*, *Scorpaena guttata*, *Citharichthys sordidus*, respectively) were collected by hook-and-line from the zone of initial dilution (ZID). This zone represents a region extending 60 m laterally from both sides of the diffuser section of the outfall of Orange County Sanitation Districts (OCSD), Southern California. White croaker (*Genyonemus lineatus*) samples from each of the three different locations, T1-near outfall, T2-midshelf and T0-inshore, at 0.3, 2.5, and 5.0 km, respectively, from the OCSD outfall were also analyzed (Fig. 1). The white croakers were collected with a Marinovich otter trawl [15]. Individual fish were weighed, measured and wrapped in clean aluminum foil and frozen. Muscle tissues were dissected from partially thawed individuals using organically-clean tools in a shore-based laboratory. Tissue samples were stored frozen in precleaned glass jars.

Partially thawed fish tissue samples (wet mass 5–15 g) were spiked with 1-phenyldodecane as surrogate standard and homogenized with a Tissum-

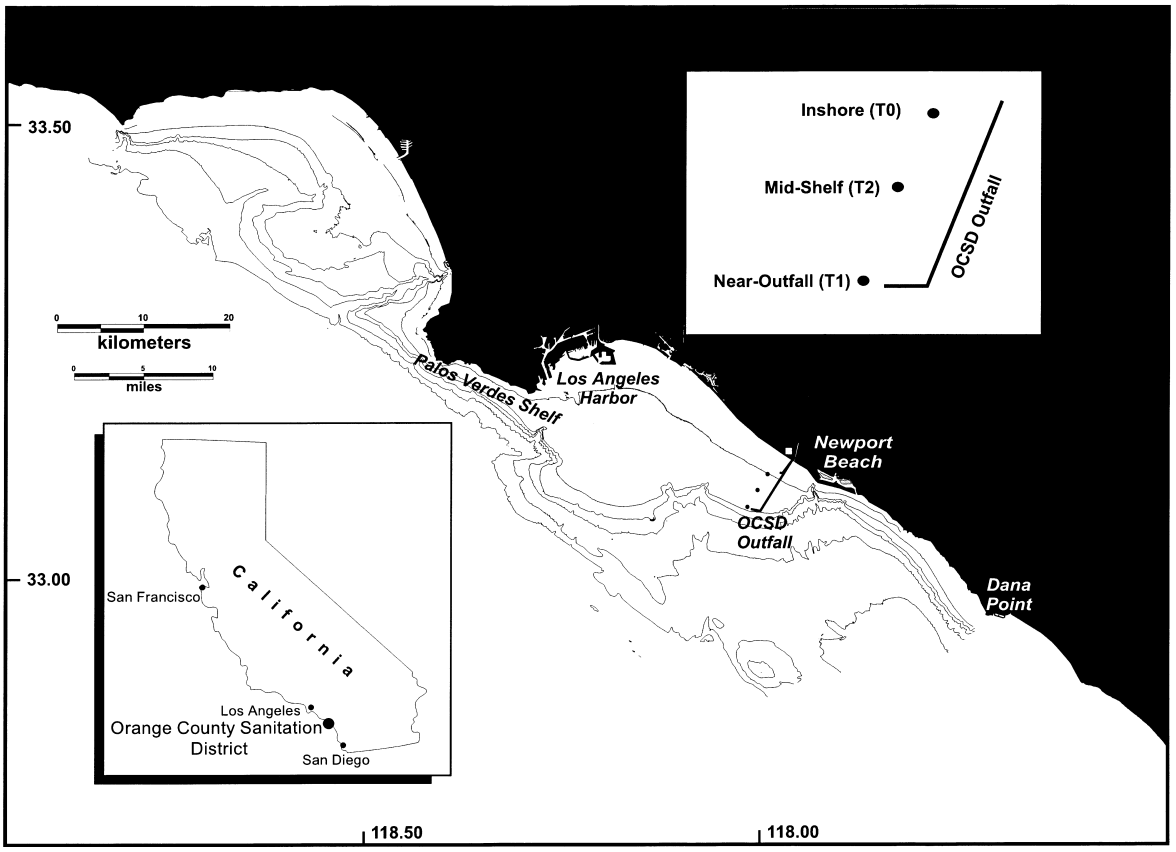

Fig. 1. Map of sampling locations near the OCSD outfall in Southern California.

izer at medium speed for 1 min and then extracted with 30 ml of dichloromethane for 2 min. The supernatant was decanted into an erlenmeyer flask and the extraction was repeated two more times. The solvent phase was percolated through an anhydrous sodium sulfate column of 3 cm wide and 10 cm long. The column was then rinsed twice with solvent to ensure complete removal of the organic compounds from sodium sulfate. The solvent was concentrated to 2 ml in a rotary evaporator at 40°C and charged to the GPC column via a glass syringe of 2 ml capacity fitted with a glass fibre filter as described above.

2.4. Analyses

The GPC column was first calibrated with the standard mixture prepared as above. Cut points from GPC elution were determined with the solvent flow at about 5 ml/min. The elution volume of dichloromethane for the LAB fraction was calibrated by running 1-phenyldodecane and a mixture of the LAB standard through the GPC and monitoring the UV absorbance at 254 nm. Discrete volume cuts were run in a gas chromatograph (Varian 3400) with column specifications and chromatographic conditions as above for GC–MS to calibrate the elution volume. The first fraction of 270 ml eluting the biolipids (i.e. matrix interference as indicated by the elution of corn oil and phthalate from the standard mixture) was rejected. All of the LABs eluted in the next 50 ml along with some of the PAHs from field samples, free of biolipid components and trichlorophenol eluted in the third and last fraction.

The LABs fraction obtained from field samples by the above GPC clean-up was then concentrated to about 1 ml in a rotary evaporator as above. It was

then blown down to near dryness in a 2-ml vial under a stream of high purity nitrogen and made up to 100–200 μl in dichloromethane and 1–1.5 μl was subjected to GC–MS analysis. GC–MS data were collected in the mass range of 50–550 amu at full scan mode. The extracted ion profiles at m/z 91 and 105 were used for quantitation of LABs by the internal standard method as described by Eganhouse et al. [1]. Here, the recovery loss of the target compounds from the matrix spike, processed concurrently with the field samples, is built into the computation for quantitation of LAB concentrations in the field samples as follows. Two aliquots of pre-extracted muscle tissue of barred sand bass (*P. nebulifer*) were spiked with a known concentration of 1-phenyldodecane as the surrogate as well as the LAB calibration standard solution and were analyzed in the same manner as the field samples. Results of the field samples were compared to the results of the two processed calibration standards, and individual LABs were quantitated based on the concentration of the average recovered standard compounds. This method has so far been used for quantitation of LABs in sediment samples [1,16] and we now apply it to fish samples as well. Total LAB concentrations represent the sum of 22 isomers listed in Table 1. I/E is the ratio of internal to external phenyldodecane (C_{12}) isomers. The method detection limit ranged from ~0.5 to 1.5 ng/g for the individual isomers based on wet mass of the tissue.

2.5. Quality assurance (QA)/quality control (QC) measures

Initially several matrix spike analyses were conducted using a spiked fish composite (barred sand bass, *Paralabrax nebulifer*) to determine the recovery and reproducibility of the technique (Table 1). No US National Institute for Standards and Technology (NIST) reference material was available for comparing LAB determination. Two matrix spikes (processed standard samples) as above and a procedure blank were analysed along with field samples. Duplicate analyses of two fish (field) samples were also performed to check the reproducibility of the method (Table 2). Due to limited sample availability no other duplicate analyses including that from midshelf region could be conducted.

3. Results and discussion

Reference material from NIST, such as fish tissue with certified values of LABs, are presently unavailable. We, therefore, evaluated method performance from QA/QC data, comparisons with results from previous studies, and comparisons among and within species collected near the OCSD outfall in LAB concentrations and homolog ratios. Spatial patterns relative to outfall in one species (white croaker) were then evaluated.

The results from the spiked tissues are presented in Table 1. The recovery of the target analytes in the matrix spikes ranged from 60 to 132% except for one compound which was recovered at about 40%. The recovery was consistently reproducible for all compounds and the relative standard deviation was within a narrow range between 1.5 and 7.6%. Only one value (12%) fell outside of this window.

A comparable amount of the extract from the sample was analyzed by GC–MS before and after GPC. Fig. 2 presents the mass chromatograms of m/z 91 and 105 of the LABs before and after the GPC procedure of a muscle tissue sample (sample GL5) as an example. The results demonstrate that GPC clean-up has removed a significant amount of matrix interference from the total extract, thereby increasing the sensitivity of detection generally by 2 to 5 times relative to that before the GPC step. GC–MS of extracts (not cleaned by GPC) fouled the injector liners and degraded the GC column very quickly (i.e. after 4–5 injections) leading to bad tailing of chromatographic peaks. Further, the extract before GPC clean-up was like a gel and had to be diluted with solvent and centrifuged for about 30 min with anhydrous sodium sulfate to obtain a reasonably clear aliquot for GC–MS injection. An attempt was also made to separate LABs from a portion of this aliquot by using silica gel column chromatography. However, anomalously high amounts of fatty acids coeluted in the aromatic fraction, containing LABs and PAHs, which interfered with the resolution and quantitation of target compounds by GC–MS. But GPC eliminated these problems of biological matrix

Table 1
Percent recovery of spiked standards of LABs from barred sand bass (*P. nebulifer*) muscle tissue

LAB	XSPIKE 1 (%)	XSPIKE 2 (%)	XSPIKE 3 (%)	XSPIKE 4 (%)	XSPIKE 5 (%)	Mean (%)	RSD (%)
6-C_{11}	67.9	63.7	64.6	69.0	69.2	66.9	2.6
5-C_{11}	57.0	41.1	49.0	59.5	57.2	52.8	7.6
4-C_{11}	62.3	60.0	65.0	66.3	68.5	64.4	3.3
3-C_{11}	62.8	61.6	63.2	69.3	67.0	64.8	3.2
2-C_{11}	61.0	63.9	64.8	65.9	64.9	64.1	1.9
6-C_{12}	64.9	63.1	66.1	69.0	69.0	66.4	2.6
5-C_{12}	68.5	66.4	69.3	70.2	69.4	68.8	1.5
4-C_{12}	63.0	68.7	69.9	71.6	72.0	69.0	3.6
3-C_{12}	57.8	69.1	60.2	69.7	67.1	64.8	5.4
2-C_{12}	63.2	63.3	65.3	66.6	67.2	65.1	1.8
7-6/C_{13}	64.9	67.4	68.3	70.2	69.3	68.0	2.1
5-C_{13}	56.2	64.3	68.4	69.3	68.5	65.3	5.5
4-C_{13}	68.1	69.7	66.0	71.7	70.5	69.2	2.2
3-C_{13}	65.8	63.9	68.4	72.9	73.8	68.9	4.3
2-C_{13}	63.3	70.0	71.0	74.4	73.3	70.4	4.3
7-C_{14}	91.5	125	113	110	103	108	12
6-C_{14}	75.9	64.2	76.9	77.9	79.1	74.8	6.1
5-C_{14}	85.5	84.1	86.8	89.0	89.0	86.9	2.1
4-C_{14}	101	97.3	100	96.4	99.9	98.9	1.9
3-C_{14}	108	108	107	103	106	106	2.1
2-C_{14}	132	129	126	122	124	127	4.0

thus resulting in a much cleaner and better resolved chromatogram as shown in Fig. 2. GPC also eliminated the need for additional solid-phase extraction or silica gel column chromatographic clean-ups.

Table 2 presents the concentrations of LAB homologs in the three different fish species as well as in the white croaker samples. Duplicate analyses of selected white croaker samples presented in Table 2 demonstrate the reproducibility and reliability of the entire methodology. The total concentrations of LABs are expressed in terms of the fish tissue wet mass as well as lipid content. The exposure conditions of fish (white croaker) from T1 are expected to be the same as those from ZID where the other fish were caught [15]. However, the lipid-normalized LAB concentrations in the three different fish species from the outfall location were uniformly lower than those in the white croaker tissues from the same location possibly reflecting a difference in their diets [15]. For example, Chub mackeral and Pacific sanddab are generalist feeders and consume small fish and planktonic crustaceans, while California scorpionfish eat fish, shrimp and small crabs. White croaker is an indiscriminate substrate forager usually feeding on infauna and epibenthic invertebrates [17].

Few other comparative data on LABs from fish are presently available like the quantitative LAB data from the fish (carp) tissues from near a carbonless paper manufacturing plant on the Upper Fox River which used a LAB mixture as a diluent. The carp tissues contained LABs at the level of 940 μg/g lipid while the river sediments contained about 30 μg/g dry mass of LABs. Both the white croaker tissue and the sediments near the OCSD outfall contain about 50 times lower than these values suggesting similar proportionality in the fish tissue and sediment LAB contents (Table 2 and Ref. [16]).

The C_{12} and C_{13} homologs are dominant in all the samples. The C_{11} homolog is present in minor amounts while the C_{14} homolog is depleted in most of the samples. The relatively high levels of the C_{14} homolog in the inshore samples may not be representative of the general trend of the location since their values approach analytical detection limits. The LABs distribution pattern in these samples is similar to those in the liver tissues of fish from the Mediterranean, also depleted in the C_{14} homologs [5]. An enrichment of shorter chain LABs in the aqueous

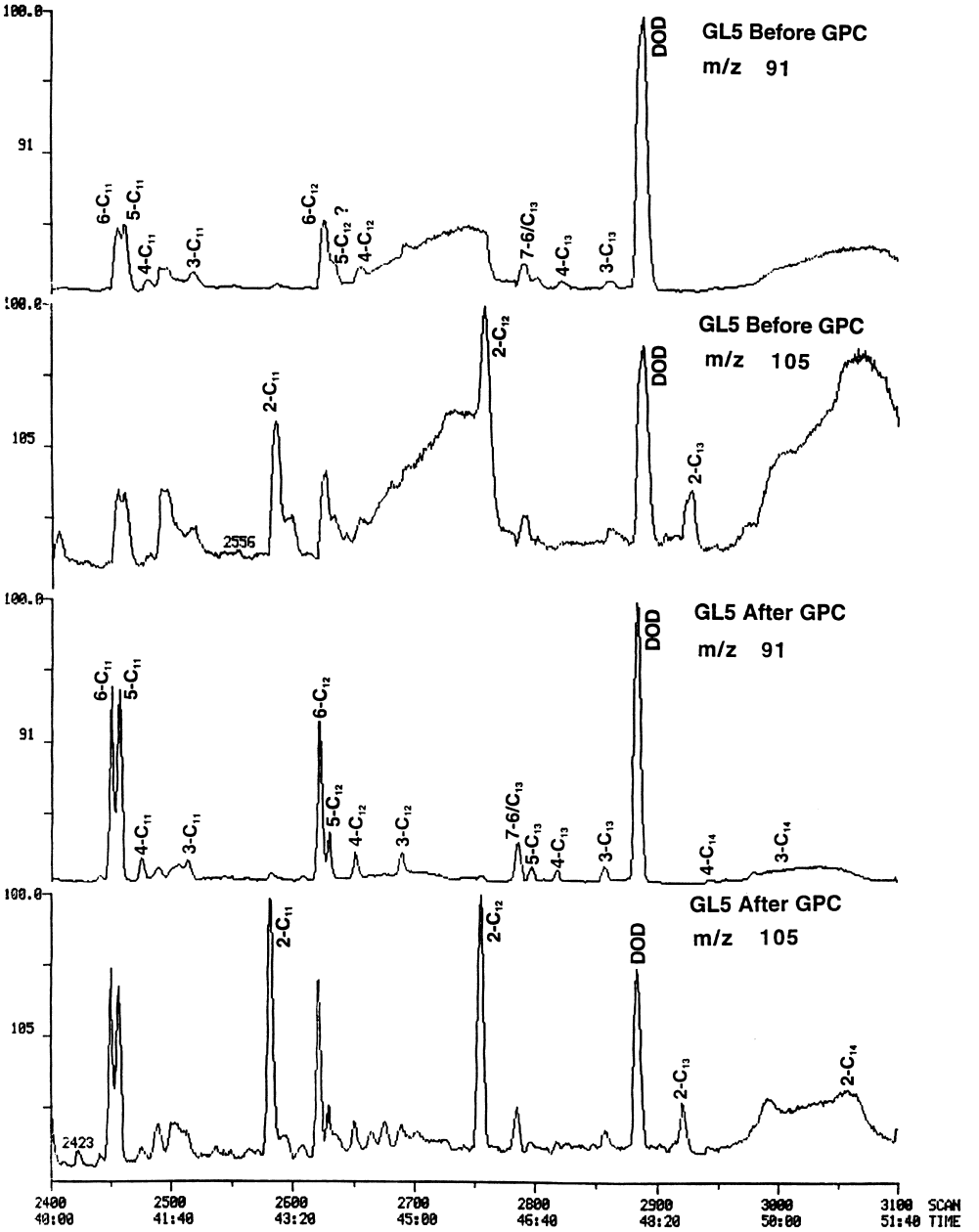

Fig. 2. Example of mass chromatograms of m/z 91 and 105 depicting LAB distributions in a white croaker sample before and after gel permeation chromatography. LABs are identified by their carbon number and position of the phenyl ring in the alkyl chain as in the tables. DOD is 1-phenyldodecane which is the surrogate recovery standard. Time scale in minutes.

phase of the effluent samples relative to the probable source material was also observed by Eganhouse and Kaplan [18]. If fish ingest LABs by absorption from the dissolved phase, then, they can be expected to contain lower proportions of higher homologs which are less soluble [6]. Selective metabolism and/or excretion may also result in depletion of the higher homologs.

Table 2
LABs in fish muscle tissue after gel permeation chromatography and gas chromatography–mass spectrometry

	S. japonicus (Chub mackerel)	*C. sordidus* (Pacific sanddab)	*S. guttata* (California scorpionfish)	*G.lineatus* (White croaker)							
				Near outfall			Mid shelf		Inshore		
	ZID[a]	ZID	ZID	T1	T1	T1	T2	T2	T0	T0	T0
	SAI00031	SAI00032	SAI00033	GL5	GL14	GL14 (dup.)	GL1	GL9	GL20	GL22	GL22 (dup.)
6-C_{11}	7.3	nd	nd	44.7	26.5	28.9	1.6	8.8	nd	nd	nd
5-C_{11}	12.3	1.5	1.1	62.8	43.8	45.5	4.6	12.6	nd	nd	nd
4-C_{11}	8.4	1.1	0.7	7.2	7.3	6.5	tr	tr	nd	nd	nd
3-C_{11}	13.9	nd	nd	6.7	13.4	5.3	5.7	tr	nd	nd	nd
2-C_{11}	11.8	2.0	1.0	14.2	16.1	14.2	3.0	3.5	nd	2.1	1.9
6-C_{12}	19.7	3.6	2.3	93.0	82.6	62.8	4.7	22.1	nd	nd	nd
5-C_{12}	18.1	3.7	2.1	23.5	25.1	15.6	nd	5.5	nd	nd	nd
4-C_{12}	15.3	2.6	2.1	11.7	13.5	11.8	1.8	tr	nd	nd	nd
3-C_{12}	14.5	4.4	4.2	15.1	21.2	28.7	3.1	3.3	nd	nd	nd
2-C_{12}	17.4	1.0	2.2	18.8	22.7	16.8	1.7	4.5	nd	nd	nd
7-6/C_{13}	23.1	5.9	3.7	25.7	27.2	18.2	8.9	6.6	nd	nd	nd
5-C_{13}	16.6	3.9	2.5	9.4	11.7	7.4	tr	tr	nd	nd	nd
4-C_{13}	13.1	3.6	2.0	5.5	8.7	8.9	nd	tr	nd	nd	nd
3-C_{13}	11.4	3.9	2.0	7.6	9.7	5.9	tr	3.2	nd	nd	nd
2-C_{13}	17.8	0.3	2.8	4.8	14.6	14.6	2.6	3.8	nd	nd	nd
7-C_{14}	34.9	nd	nd	tr	nd	nd	nd	nd	nd	nd	nd
6-C_{14}	nd	nd	nd	tr	tr	tr	nd	nd	nd	nd	nd
5-C_{14}	nd	nd	nd	tr	tr	tr	1.2	nd	nd	nd	nd
4-C_{14}	nd	nd	nd	2.7	4.4	6.0	tr	2.4	nd	tr	1.6
3-C_{14}	nd	nd	nd	6.2	11.3	10.8	nd	tr	4.4	nd	nd
2-C_{14}	1.1	nd	nd	2.6	6.0	3.6	2.9	0.4	nd	nd	nd
Total (ng/g wet)	257	37.5	28.8	362	366	311	42.0	76.8	4.4	2.1	3.5
Total (μg/g lipid)	12.8	12.5	7.2	16.5	18.6	22.6	2.8	4.7	0.4	0.2	0.4
I/E[b]	0.80	0.92	0.51	2.60	1.87	1.37	0.72	3.55	nd	nd	nd

[a] Zone of initial dilution; nd, not detected, below MDL; tr, trace amounts, slightly >MDL, but not quantifiable.
[b] Ratio of (6-C_{12}+5-C_{12}) to (4-C_{12}+3-C_{12}+2-C_{12}).

The I/E values have been used as a measure of biodegradation of the external compared to the internal isomers of phenyldodecane [19]. Consequently, LABs not only reflect the potential impacts of wastewaters, but also on the degree of microbial degradation of wastewaters. The I/E values of white croaker are about 4–6 times greater than that from the other three fish species which may again be attributed to species-related LAB metabolism and/or dietary constituents. Despite the possible metabolism of LABs by fish [11], the total content of LABs in the white croaker species apparently correlates with their proximity to the sewage discharge, which is their point source, as discussed below.

The total LABs content in the white croaker collected from varying distance from the outfall also corroborates with the sediment LABs concentration measured by Phillips et al. [16] and in the sediments investigated from the same region more recently (Orange County Sanitation District, CA, USA, unpublished data). The sediment samples near the outfall contain the maximum, and the midshelf samples contain lower levels, while the inshore samples are rather depleted in LABs. This gradient in the distribution is consistent with their probable source in the sewage effluents from OCSD. This same trend is exhibited in the LABs from the muscle tissues of white croaker [15]. The LABs content in muscle tissue apparently reflects chronic inputs from the outfalls and suggest that they could serve as a monitoring tool to track exposure of fish to sewage effluents. It is believed that white croaker would be a suitable candidate for such assessments, since it is a bottom-dwelling fish commonly found near the

OCSD outfall. Prior studies in the southern California coastal regions, including in the vicinity of sewage outfalls, have reported that this species was impacted by anthropogenic contaminants. Reproductive impairment and liver lesions of this species have been associated with adverse effects of exposure to toxic chemicals in the southern California coastal environment [20,21]. Gel permeation chromatographic clean-up developed here for the first time for concentrating LABs in fish could certainly be applied to other biological tissues in any exposure studies. This method, after suitable modifications, can further be translated to high-performance liquid chromatography which would require much lower volumes of organic solvent to recover the target analytes and shorten the sample processing time as well.

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