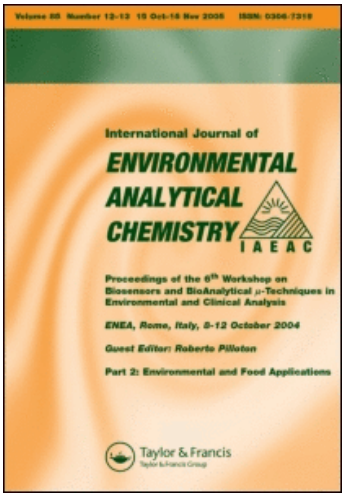

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Effect of Biogenic Impurities on Polycyclic Aromatic Compounds Analysis by Fluorimetry

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To cite this Article Hellou, Jocelyne and Upshall, Catherine(1993) 'Effect of Biogenic Impurities on Polycyclic Aromatic Compounds Analysis by Fluorimetry', International Journal of Environmental Analytical Chemistry, 53: 4, 249 — 259
To link to this Article: DOI: 10.1080/03067319308044429
URL: http://dx.doi.org/10.1080/03067319308044429

Intern. J. Environ. Anal. Chem., Vol. 53, pp. 249-259
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EFFECT OF BIOGENIC IMPURITIES ON POLYCYCLIC AROMATIC COMPOUNDS ANALYSIS BY FLUORIMETRY

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(Received, 16 September 1992; in final form, 15 October 1992)

Polycyclic aromatic compounds (PAC) are ubiquitous in nature. The International Oceanographic Commission (IOC) has recommended their analysis in environmental samples, by ultra-violet/fluorescence (uv/f) spectrophotometry. In the present investigation, the effect caused by the presence of biogenic impurities on the perceived PAC concentration of anthropogenic solutions was examined. Lipid extracts of the foot of the clam, *Mya arenaria*, the muscle tissue of cod, *Gadus morhua* and of the harbour porpoise, *Phoceana phoceana*, as well as squalene represented the biogenic material. Venezuelan crude, no.2 fuel oil and chrysene were chosen as the PAC solutions, representing environmental extracts. Results indicate that more than 30–100 ng/µl or 0.045–0.150 mg/g (dry weight) of lipid extracts are needed to start to affect (>5–10%) the perceived PAC concentrations, while the amount of squalene needed is over 370 ng/µgl or 0.555 mg/g (dry weight). The latter is above levels reported in tissues of most marine animals, except sharks. This supports the value of fluorimetry as a relatively inexpensive screening technique for monitoring PAC levels in marine organisms. However, it does not diminish the importance of analysing samples by gas chromatography-mass spectrometry to obtain more detailed information.

KEY WORDS: Fluorimetry, PAH, hydrocarbons, squalene, muscle, lipids.

INTRODUCTION

Ultra-violet/fluorescence (uv/f) spectrophotometry has often been used in the analysis of polycyclic aromatic hydrocarbons (PAH) extracted from water[1], sediments[2], crude oil[3], whole marine organisms[4], tissues of animals[5] or urine[6]. It is important to point out that uv/f analysis does not discriminate between PAC (containing heterocyclics) and PAH, therefore, the term PAC is preferable when referring to the fluorimetric analysis of aromatics. We have used fluorescence spectrometry to measure PAC levels in marine mammals from the Northwest Atlantic[7,8].

An advantage of uv/f compared to other analytical techniques is the high sensitivity of this method, when dealing with single compounds or mixtures (better than 0.1 ng/µl). The sensitivity depends on the choice of an appropriate excitation wavelength in the near

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ultra-violet region (200–400 nm) and on the choice of emission wavelength. Another advantage of this method is that total PAC rather than single PAH are quantified, regardless of their volatility. Other techniques such as gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS, electron impact, EI, in the single ion monitoring mode, SIM or total ion chromatogram, TIC) do not always meet these detection limits, especially when dealing with mixtures. Also, in order to maintain the detection limits, regular clean up of the GC-MS instrumentation, which can be time consuming, is a must. GC related analyses are also limited by the volatility of the components that can be examined. High performance liquid chromatography (HPLC) coupled to a uv/f detector represents a step ahead of uv/f alone, since chromatography allows the separation of the components prior to their detection. A disadvantage of the latter two methods is that a variety of columns, temperature programs or solvents and wavelength can be used. Consequently, more variables are introduced which complicates comparison of results obtained from different laboratories. Also, as with uv/f alone, the detection of unknown PAC by HPLC-uv/f requires the use of an alternate spectroscopic technique (MS) to identify the unknowns. As with GC-MS in the SIM mode, the availability of standards limits the number and type of PAC that can be analyzed by HPLC. The analysis of environmental samples with different chromatographic and/or spectroscopic techniques (LC-uv/f vs GC-MS; GC vs uv/f) has been compared in individual laboratories[9, 10] and through intercalibration exercises[11–16].

A concern regarding the quantification of PAC using uv/f spectrophotometry is the effect of interfering biogenic material present in extracts. Quenching or enhancement of the fluorescence can take place when unsaturated non-PAC components are present in a mixture. This problem can be more or less pronounced depending on the type of sample analyzed and the chromatographic steps preceding the analysis.

In the present study, we examined the effect caused by the presence of biogenic 'impurities' on the quantification of the anthropogenic content of PAC extracts. Three anthropogenic type solutions were used: Venezuelan crude oil, no.2 fuel oil and chrysene. Different types of biogenic solutions, reflecting the kind of impurities that can be present when analyzing muscle tissue of marine organisms were chosen: lipid extracts from the foot tissue of clam, muscle tissue of cod and of harbour porpoise, and squalene. The anthropogenic solutions were made up at two concentrations, near detection limit and approximately twenty times higher, well below concentrations that would be expected to create quenching[3, 17]. Spiking with biogenic type material was performed over a wide range of concentrations. This was done to determine when impurities start to affect the perceived PAC concentrations. Results were expressed in terms of four different pairs of excitation and emission wavelength corresponding to chrysene, phenanthrene, Venezuelan crude and no.2 fuel oil. The choice of chrysene as a reference material has been recommended by the International Oceanographic Commission (IOC). Ideally, the excitation/emission wavelengths corresponding to the anthropogenic material should be used[10]. However, other wavelength were also chosen in the present investigation, to allow comparison.

MATERIALS AND METHODS

A Perkin-Elmer LS-5 spectrophotometer equipped with a xenon lamp was used for uv/f analyses. Solutions were prepared in double distilled hexane (3 ml) and placed in a rectangular quartz cell (1 × 1 cm). Readings were obtained with a 5 nm slit-width. The anthropogenic solutions representing a mixture of PAC, Venezuelan crude and no.2 fuel oil were analyzed by Seakem Oceanography Ltd, Sidney, British Columbia, Canada. Results of the analyses of 22 aromatic compounds, PAH and heterocyclic aromatics (dibenzothiophenes: DBT) are presented in Table 1. Analysis by synchronous fluorescence (25 nm offset) displayed a maximum exitation/emission wavelength pair at 280/305 nm and 335/360 nm, for no.2 fuel oil and Venezuelan crude, respectively. The use of synchronous fluorescence in the analysis of PAH has been discussed previously[18, 19]. These two wavelength pairs were used for the calibration curves as well as 310/360 and 313/374 nm, in reference to chrysene and phenanthrene, respectively.

Table 1 Approximate concentration of aromatics (μg/g).

Compounds	*Venezuelan crude*	*No.2 fuel oil*
C-2 benzene (B)	>11000	>11000
C-3 B	2500	5000
C-4 B	400	1700
C-5 B	690	3600
naphthalene (NA)	>410	>1500
C-1 NA	>1900	>6400
C-2 NA	3800	10000
C-3 NA	4400	11000
C-4 NA	670	2100
fluorene (F)	70	200
C-1 F	130	530
C-2 F	180	600
dibenzothiphene (DBT)	170	180
C-1 DBT	830	620
C-2 DBT	1200	750
C-3 DBT	300	160
C-4 DBT	120	31
phenanthrene-anthracene (PA-A)	260	390
C-1 PA-A	1100	1100
C-2 PA-A	1000	970
C-3 PA-A	470	290
C-4 PA-A	180	91
Σ 22 hydrocarbons	31,780	58,212

-Analyses of the oils were performed by Seakem Oceanography Ltd, Sidney, British Columbia, in 1985, the company is now known as Axys Analytical Services Ltd.

Lipids from the muscle tissue of the harbour porpoise, *Phocoena phoceana*, cod, *Gadus morhua* and clam, *Mya arenaria* were extracted according to the Bligh and Dyer method[20]. A Hewlett-Packard 5890 gas chromatograph (GC) coupled to a Hewlett-Packard Series 5890 mass selective detector and a Hewlett-Packard Series 300 data system, equipped with a CP-Sil-5 column (25m × 0.20mm i.d.) were used to analyze the lipid content of the tissues. The temperature program started at an initial temperature of 80°C, maintained for 1 min, raised at a rate of 4°C/min., up to a final temperature of 250°C, where it remained for 15 min.

RESULTS AND DISCUSSION

Results of the fluorimetric analyses are presented graphically, in Figures 1 to 4, to give a good numerical and visual appreciation of the effect caused by the presence of biogenic impurities (x axis) on the fluorescence of anthropogenic PAC, expressed in terms of the perceived PAC concentrations (y axis). In what follows, the effects are discussed first, in terms of the same reference material as the anthropogenic solutions. Later in the discussion, we compare the results obtained using other reference materials.

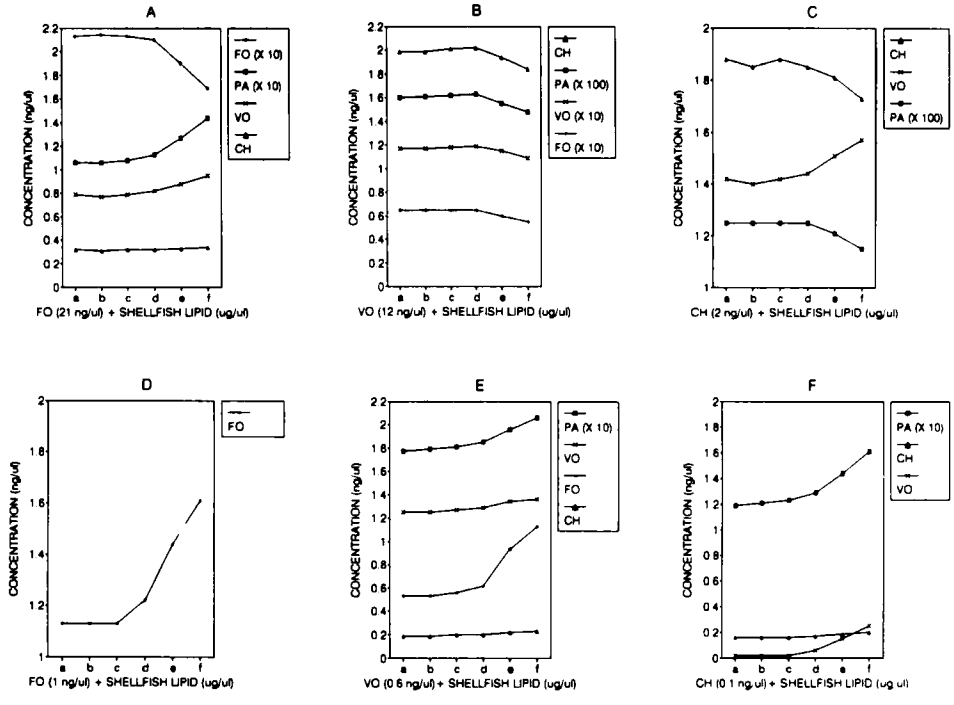

Figure 1 Effect of shellfish lipids on the perceived PAC concentration of anthropogenic solutions. PA, CH, FO and VO represent phenanthrene, chrysene, no. 2 fuel oil and Venezuelan crude. Subscripts a, b, c, d, e and f represent 0, 0.0003, 0.004, 0.03, 0.15–0.17, 0.30–0.33 μg/μl.

The GC-MS chromatograms obtained for the muscle tissue extracts showed the presence of a large number of compounds. The fatty acids composition of the lipid extracts has been previously reported for clams[21] and for cod[22] where a variety of unsaturated components, with 1 to 6 double bonds, were identified. Similar unsaturated fatty acids have also been detected in various tissues of marine mammals[23, 24].

The effects caused by the presence of lipids on the analysis of PAC extracts by fluorimetry were investigated first. Addition of the lipid extract obtained from the foot of the clam, *Mya arenaria* to relatively concentrated solutions of no.2 fuel oil (20 ng/μl), Venezuelan crude (12 ng/μl) and chrysene (2 ng/μl) produced only a slight decrease in the perceived PAC concentration (Figure 1A, B and C). Addition of 0.03 to 0.3 μg/μl of lipid extract resulted in a 10 to 20% decrease in perceived PAC concentrations. Addition of the clam extract to more dilute anthropogenic solutions (1.2, 0.6 and 0.1 ng/μl, of fuel oil, Venezuelan and chrysene, respectively) produced an increase in the perceived PAH concentrations (Figure 1D, E and F). This increase was negligible (<10%) with Venezuelan crude and chrysene, but more pronounced in the case of the fuel oil (from 5 to 50%, when adding 0.03 to 0.3 μg/μl).

Addition of the extract obtained from the muscle of cod, *Gadus morhua* to concentrated PAC solutions resulted in a very small (<10%, Figure 2A, B and C) decrease in the perceived PAC concentration after the addition of up to 0.3μg/μl of lipid extract. In the case of more dilute PAC solutions (Figure 2D, E and F) increases of 2 to 50% in the PAC concentrations

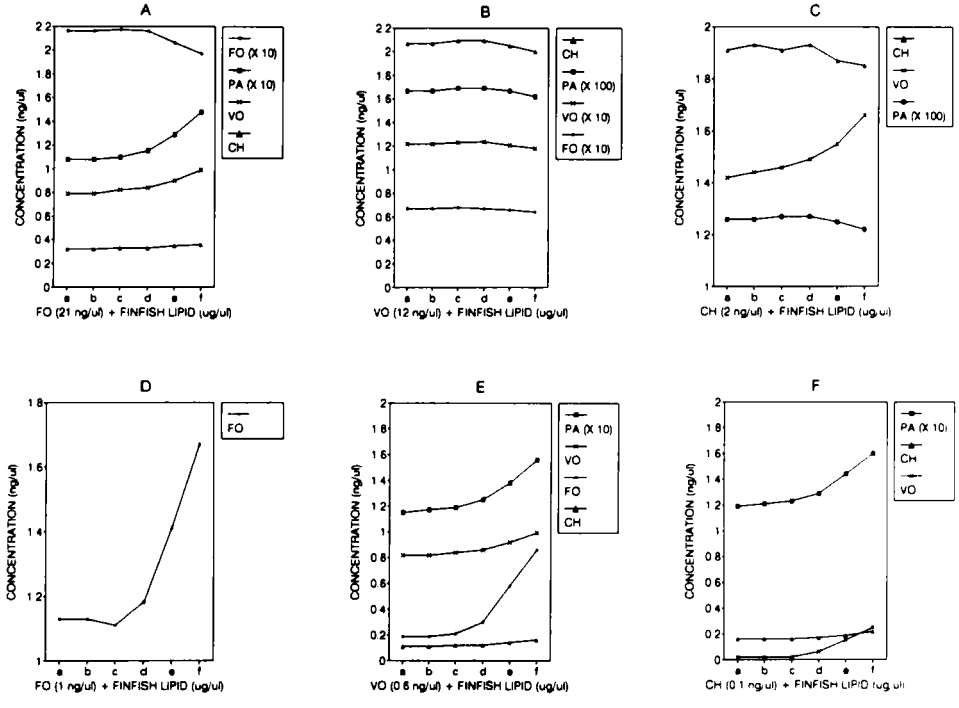

Figure 2 Effect of finfish lipids on the perceived PAC concentration of anthropogenic solutions. PA, CH, FO and VO represent phenanthrene, chrysene, no. 2 fuel oil and Venezuelan crude. Subscripts a, b, c, d, e and f represent 0, 0.0003, 0.003–0.004, 0.03, 0.15–0.16 and 0.29–0.31 μg/μl.

were observed after the addition of 0.03 to 0.3 μg/μl of lipid extracts. As with the above clam results, the effect was more pronounced when spiking the fuel oil.

The addition of the extract obtained from the muscle of harbour porpoise, *Phoceana phoceana* to the three concentrated anthropogenic solutions resulted in a negligible change (5%) in the perceived PAC concentrations (Figure 3A, B and C). The effect of biogenic impurities was again more pronounced with the dilute anthropogenic solutions. As previously observed, a much larger increase was perceived with the fuel oil (up to 150%) than with the other two solutions (5%, Figure 3E and F vs D).

The lipid concentrations expressed in terms of volume can be translated into concentrations in terms of analyzed animal tissue. If we assume that the present results refer to the extraction of 2g (dry) of muscle tissue, and since 3000 μl of solvent were used, then we can transform the μg/μl values into μg/g. The lipid concentrations that would 'affect' the perceived PAC concentration, on average >0.1 μg/μl would become 150 μg/g. This amount of lipid would be detected by infra-red (IR) spectroscopy, where a prominent characteristic band due to a carbonyl group would appear; or by GC-MS analysis of the extracts, since some of the fatty esters represent more than 10% of the total components[21–24].

The effect caused by the addition of a single unsaturated component to the hydrocarbons mixture was investigated next. Squalene is a naturally occurring terpenoid found in biological

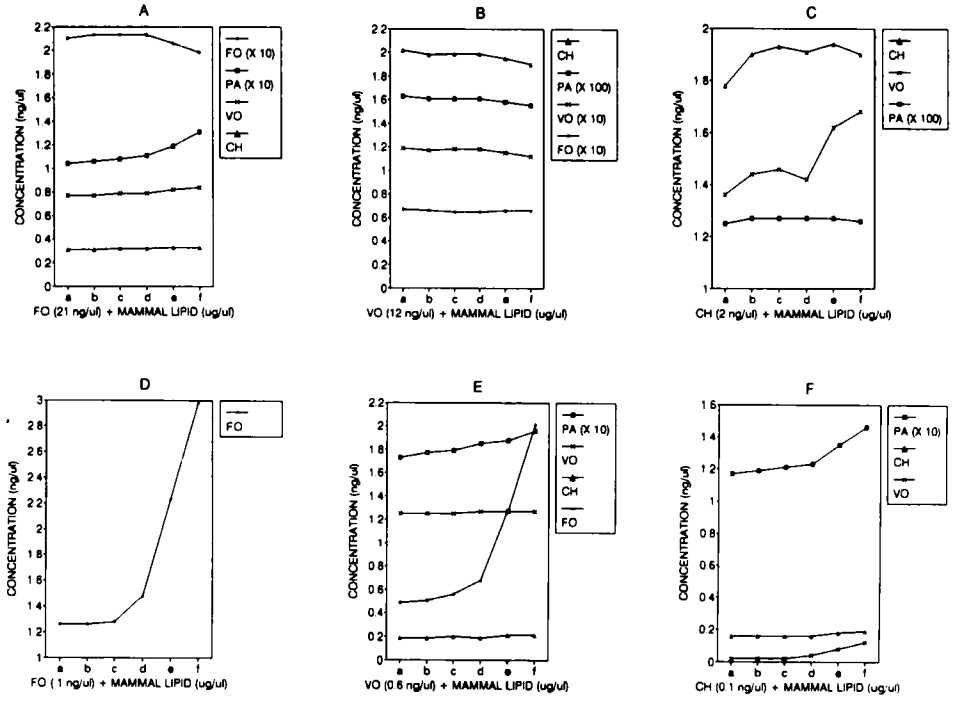

Figure 3 Effect of marine mammals lipids on the perceived PAC concentration of anthropogenic solutions. PA, CH, FO and VO represent phenanthrene, chrysene, no. 2 fuel oil and Venezuelan crude. Subscripts a, b, c, d, e and f represent 0, 0.0003, 0.003, 0.03, 0.15–0.17 and 0.30–0.33 μg/μl.

Table 2 Concentration of squalene (μg/g) in tissues of marine organisms.

Animal	*Species*		*Wet weight*
crabs[25]	*Cancer magister*		0.2–1.6
worms[25]	*Nereis vexollossa*		0.5–6.6
limpets[25]	*Collisella pelta*		0.2–7.6
clams[25]	*Macoma balthica*		0.1–8.1
mussels[25]	*Mytilus edulis*		0.2–12.0
sharks[26]	*Dalatias licha,*	liver	620×10^3
		muscle	550
		ovary	58×10^3
sardines[27]	*Sardina pilchardus,*	muscle	1.8–4.1
		liver	1.4–30.6
		gills	1.1–3.9
		gonads	0.2–1.6
		gastric content	5.0–14.4
finfish[32]	7 species,	liver	68
		skin	64
		dark muscle	60
harbour porpoise[33]	*Phoceana phoceana,* (mature males)	muscle	10–50[a]
		liver	2–10
		kidney	2–16

a: A dozen muscle samples were analyzed and 3 samples of liver and kidney.

tissues of living organisms (Table 2). It is the biochemical precursor of cholesterol and of the bile salts. Squalene is present at different concentrations depending on the tissue and animal species studied[25,26]. It can represent the major component in PAC extracts[25,27] and is not usually removed by column chromatography over alumina or silica.

For a higher concentration of Venezuelan crude (12 ng/μl), no.2 fuel oil (21 ng/μl) and chrysene (2 ng/μl), there was a negligible effect (<5%) caused by the presence of squalene, added in relatively large amounts (up to 0.8–0.9 μg/μl), at the four wavelength pairs used (Figure 4A, B and C). For the lower concentration of these same three anthropogenic solutions (0.6, 1.3 and 0.1 ng/μl, respectively), effects similar to those reported above were observed (Figure 4D, E and F). When squalene was added at a concentration higher than 100 ng or 0.1 μg/μl, in excess of 100 times that of the anthropogenic material, the estimated PAC concentration started to be affected. These changes can be examined according to the wavelength pairs of the 4 reference materials used.

Squalene (up to 0.9 μg/μl) added to dilute no.2 fuel oil showed a maximum increase of 26% in the perceived PAC concentration (Figure 4D). Once again, as observed with the other spiked no.2 fuel oil solutions (Figures 1D, 2D and 3D) compared to the Venezuelan and chrysene anthropogenic solutions, the uv/f could only be measured in terms of the oil itself. The addition of squalene (370 ng/μl and 1.1 μg/μl) to dilute Venezuelan crude (0.6 ng/μl) showed an increase in the perceived PAC concentration, of 20 and 40%, in terms of chrysene; of 10 and 20%, in terms of Venezuelan crude and phenanthrene units, and an increase of 200 and 300%, in terms of no.2 fuel oil units (Figure 4E). It is of interest to report that the synchronous fluorescence spectrum of squalene (using a 25 nm offset) displayed a maximum at 280/305 nm, which is also the maximum observed for no.2 fuel oil. Spiking of

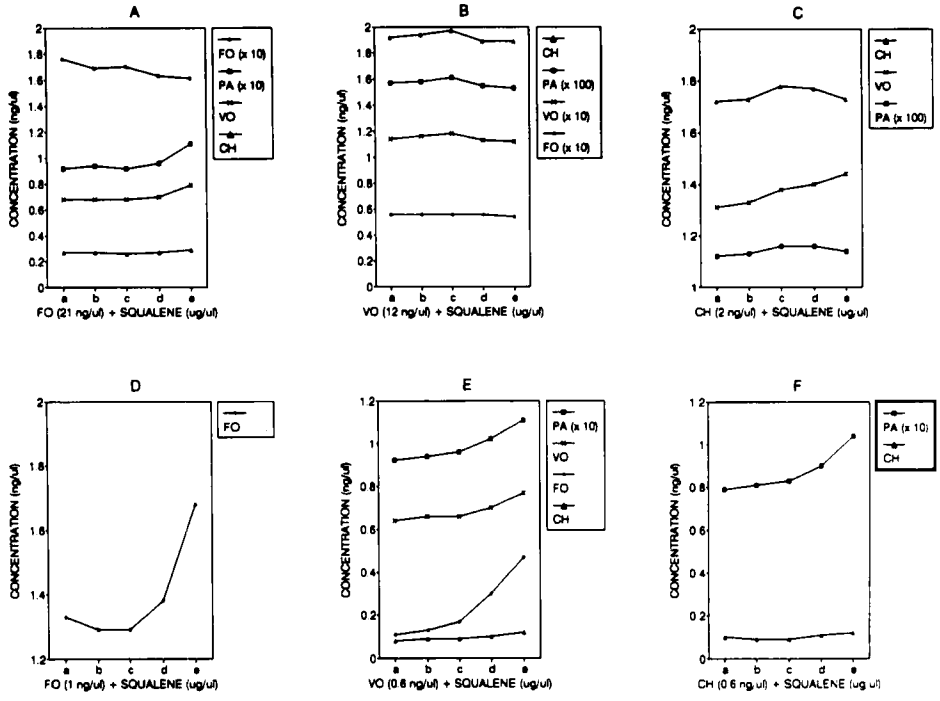

Figure 4 Effect of squalene on the perceived PAC concentration of anthropogenic solutions. PA, CH, FO and VO represent phenanthrene, chrysene, no.2 fuel oil and Venezuelan crude. Subscripts a, b, c, d and e represent 0, 0.05, 0.09–0.10, 0.33–0.36, 0.85–0.92 μg/μl.

the dilute chrysene solution with squalene (up to 0.9 μg/μl), showed a 5 and 25% increase in the perceived PAC concentration, in terms of chrysene and phenanthrene units, respectively (Figure 4F).

The effect caused by the presence of unsaturated biogenic impurities on the PAC concentration measured by uv/f, has been previously recognized when analyzing whole molluscs or the visceral mass of invertebrates[28–30]. The importance of the purity of an extract (muscle) has been numerically clarified in the present study. Regardless of the extraction method used[31], lipid derived impurities (major constituents: fatty esters or transesterified fatty acids) can also be isolated from the tissue samples during this step. After extraction, purification by column chromatography or by HPLC, using various supports is needed to isolate the hydrocarbons from the rest of the extracted material. The efficiency of the chromatographic steps is crucial in providing accurate results and its importance has been demonstrated in the present investigation. In previous analyses of muscle tissue, unsaturated non-PAC, other than squalene have not been detected. Therefore, the present results indicate that if the chromatographic purification is efficient, prior to quantification by fluorimetry, then results are representative of the presence of trace levels of aromatics in the tissues (PAH, heterocyclic aromatics and possibly benzenoids or co-eluting organochlorine contaminants). The amount of squalene that starts to affect the perceived PAC concentration is >370 ng/μl

or 0.555 mg/g (dry) and above the levels observed in the majority of tissues listed in Table 2. The analysis of shark tissues would represent an exception. These fish species are somewhat unique, since it is believed that the absence of a swimming bladder in sharks is replaced by the high levels of squalene in tissues, where squalene is reported to play a role in buoyancy compensation[26].

Other observations should be made regarding the present investigation. The low and high concentrations of Venezuelan crude (0.6 and 12 ng/μl) and of no.2 fuel oil (1.3 and 20.9 ng/μl) can be expressed in terms of the sum of 22 aromatic compounds (Table 1). The concentrations would then represent 3.2 and 5.8% of the Venezuelan crude (0.02 and 3.84 ng/μl) and the fuel oil (0.08 and 1.21 ng/μl), respectively. The concentration of each of the 22 PAC would have been between 3 and 454 times lower, in Venezuelan crude and between 5 and 213 times lower, in no.2 fuel oil. It is recognized that analysis of samples by GC-MS or HPLC-uv/f would require a smaller volume of solvent (<100 or 50μl) than by uv/f (presently 3000μl), therefore 30 or 60 times more concentrated samples (1.2 to 230 ng/μl). Even then, the concentration of single components would have been below the detection limits for all the dilute solutions and for a number of the PAC (Table 1), in the case of the more concentrated solutions.

Variations of 10–20% are not unusual when performing duplicate analyses of PAC in environmental samples. Therefore most of the changes reported in the present investigation are small relative to the variability due to sample work-up. However, since there is an effect due to the presence of biogenic impurities (major components being fatty esters, minor possibly being non-PAC contaminants), our results emphasize the importance of ascertaining the purity or composition of an extract, by alternate spectroscopic methods, such as IR or GC-MS, prior to reporting uv/f results.

The good choice of chrysene as a reference material (by the IOC) is apparent. In most cases, the PAC concentration of the solutions could be measured and was least affected by the spiking with lipids and squalene. The correlation coefficient between concentrations expressed in terms of Venezuelan crude (used as the anthropogenic solution) and chrysene was better than 0.99, and between the fuel oil (also used as the anthropogenic solution) and chrysene was better than 0.97. In all cases, chrysene underestimated the PAC concentration expressed in terms of Venezuelan crude or fuel oil. It should also be noted that, as expected, measuring the PAC present in Venezuelan crude, in term of the wavelength pair characteristic of fuel oil, gave results inconsistent with those observed when using chrysene.

It is well recognized that uv/f analysis of total PAC needs to be accompanied by more detailed GC-MS analyses of components, whenever possible. The present results do not mean that GC-MS analyses of PAC are unnecessary, but that proof of their relative significance, using uv/f is important. The first technique used by itself can give questionable results, if the data is not supported by more sophisticated analytical results. On the other hand, the significance of GC-MS results (individual components) would be increased when accompanied by uv/f data (total mixture).

CONCLUSION

We have attempted to clarify the question of when a perceived PAC concentration measured in terms of uv/f will be representative of the actual amount of PAC present in muscle tissue. This was investigated by spiking hydrocarbon solutions with lipid extracts and with squalene. These types of biogenic impurities could be present in tissue extracts. The former represented incomplete or inefficient chromatography of a PAC extract, while the latter represented an unsaturated non-PAH biogenic component known to be present in animal tissues. Lipids affected the perceived PAC concentration when present in relatively high amounts (>0.03 μg/μl or 45μg/g, dry weight), easily detectable by alternate spectroscopic techniques. The biogenic material squalene caused an effect only when present in a concentration (>370 ng/μl or 0.555 mg/g, dry weight) higher than that reported to be present in most muscle tissue of marine animals, other than sharks.

Acknowledgements

This research was funded in part by the Green Plan For Toxic Chemicals and the Program For Energy Research and Development.

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