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Standard Analytical Procedures of the NOAA National Analytical Facility, 1986:
Metabolites of Aromatic Compounds in Fish Bile

by
Margaret M. Krahn
Leslie K. Moore
William D. MacLeod, Jr.

May 1986

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service

STANDARD ANALYTICAL PROCEDURES

O F T H E

NOAA NATIONAL ANALYTICAL FACILITY, 1986

METABOLITES OF AROMATIC COMPOUNDS IN FISH BILE

prepared for

The NOAA National Status and Trends Program

and

The Outer Continental Shelf Environmental Assessment Program

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Margaret M. Krahn

Leslie K. Moore

William D. MacLeod, Jr.

National Marine Fisheries Service
Northwest and Alaska Fisheries Center
Environmental Conservation Division
2725 Montlake Boulevard East
Seattle, Washington 98112

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PREFACE

Analytical procedures for estimating the exposure of fish to petroleum-related aromatic compounds from the environment have been developed over the past 7 years in our laboratories, sponsored by NOAA's Outer Continental Shelf Environmental Assessment Program (OCSEAP) with funding from Minerals Management Service. ,OCSEAP sponsors research on the fate and effects of petroleum-related chemicals on marine organisms and uses the results of this research to assess the environmental impact of gas and oil development on the marine resources of the outer continental shelf.

The analytical procedures we have developed are in their second year of use in the Benthic Surveillance Project of NOAA's National Status and Trends (NS&T) Program. The NS&T Program was begunin 1984 to document and assess the present status and future trends of environmental quality along the nation's coasts and in its estuaries. NS&T is employing a standard set of analytical methods in order to decrease the variability of the measurements. Thus, our analytical procedures are being documented in this technical memorandum.

For further information on NOAA's programs write: Outer Continental Shelf Environmental Assessment Program, NOAA/NOS/OAD/Alaska Office, Box 56, 701 C Street, Anchorage, AK 99513 or National Status and Trends Program, NOAA/NOS, N/OMA32, Rockville, MD 20852.

INTRODUCTION

Since its inception in 1976, NOAA's National Analytical Facility (NAF) has been at the forefront in developing and employing advanced methods to analyze samples from the aquatic environment for traces of toxic chemicals. These activities have focused primarily on methods for determining anthropogenic organic compounds such as aromatic hydrocarbons (AHs) and chlorinated hydrocarbons both in sediments and in organisms.

Bottom-dwelling fish often encounter high concentrations of AHs and other chemical contaminants (e.g., from spilled petroleum or industrial discharges) in polluted coastal waters and estuaries. Fish rapidly take up many of these contaminants. However, the uptake of AHs cannot usually be assessed by analyzing directly for these compounds because fish rapidly convert AHs to a variety of metabolic products. To address this issue, NAF chemists have developed analytical methods for directly measuring concentrations of metabolites of aromatic compounds in fish (Krahn et al. 1980, 1982, 1984);

If the results from one study or one site are to be compared to those from another, uniform analytical methods are required. This manual for the analysis for selected metabolites of aromatic compounds in fish bile 'was prepared to meet this need. The methods described in this publication have been applied to:

* statistically link hepatic lesions, including neoplasms, in English sole from Puget Sound to concentrations of bile metabolites which fluoresce at the benzo[a]pyrene wavelengths (Krahn et al. 1984, 1986);

* follow the exposure of sturgeon from the Columbia River to spilled oil (Krahn et al. in press),;

* monitor the environmental quality of fish from coastal waters and estuaries under the NUT Program.

In addition to the Literature Cited (page 23), an Appendix listing all of our publications concerned with this research appears on page 24.

SECTION 1

M A T E R I A L S

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MATERIALS

Disclaimer: Mention of a product or company name does not imply endorsement by the Department of Commerce to the exclusion of others that may be suitable.

Note: All chemicals and solvents must be of the highest purity obtainable.

A. Solvents

methanol	Burdick and Jackson, high purity (or equivalent)
water	Baker Analyzed Reagent (HPLC) (or equivalent)
acetic acid	Baker Ultrex (5uL/L water)

B. Columns

analytical	Perkin-Elmer HC-ODS; 25 x 0.26 cm (ID), 10 um reverse-phase, No. 089-0716
guard (precolumn)	Waters (2.3x0.39 cm);.packed with reverse-phase Vydak, (30-44 um), No. 201-SC

C. Vials

sample collection	amber, 4 mL with Teflon-lined septa, (Sunbrokers, Wilmington, NC)
autosampler	as above, with glass limited-volume inserts, compression springs, (Millipore-Waters)

D. Standards

calibration	HPLC/fluorescence calibration check solution in methanol, prepared under yellow lights, stored at-20°C in amber vials. naphthalene (NPH) (3.3 ng/pL) benzo[a]pyrene (BaP) (0.31 ng/pL) Note: Concentrations of standards may be adjusted as necessary.
bile pool (for quality assurance)	Bile is collected from enough fish from a single contaminated site to give 3 mL when pooled. The pool is then subdivided into 50 PL samples and stored at -20°C in amber vials. The bile pool is replaced when concentrations decrease, ca. every 6 mo. (See also Quality Assurance in Section 3.)

E. Gas Cylinders and Apparatus

air (for the automatic sampler)	Ohio breathing air, CGA grade E (or equivalent)
helium (for degassing solvents)	Grade 4.5 (purified, 299.995%)
regulators	2-stage (1 for each cylinder)

F. Equipment for Dissection

scissors.	stainless steel
forceps	stainless steel
scalpel with no. 11 blade	stainless steel blade
hemostats	stainless steel
wash bottle	Teflon, containing methanol

SECTION 2

INSTRUMENTS AND SPECIFICATIONS

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INSTRUMENTS AND SPECIFICATIONS

A. High-performance Liquid Chromatograph (HPLC)

Spectra-Physics Model 8000B (or equivalent) including:

temperature-controlled oven

gradient programming

recorder-integrator; dual channel (for integration and data reduction on two channels at the same time); second channel is optional.

disk memory unit, optional

B. Automatic Sampler

Millipore-Waters, WISP,Model 710A (or equivalent)

capable of reproducible 5 uL injections with relative standard deviation (RSD) < 5%

capable of sampling from small volumes (down to 20 uL)

Note: The use of the autosampler is desirable for best reproducibility, but manual injection techniques can be used when no autosampler is available.

C. Fluorescence Spectrometer(s)

Perkin-Elmer: Model MPF 44A,(or equivalent)

Model LS-4 (or equivalent) Note: Second spectrometer is optional.

Separate monochromators for excitation and emission

Wavelength accuracy of ± 2 nm.

Small-volume flow cells (3-20 uL)

Signal-to-noise ratio < 6O:l when measured on the Raman band of water with excitation at 350 nm and emission at 397 nm, 10 nm slits for both monochromators.

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SECTION 3

METHODS

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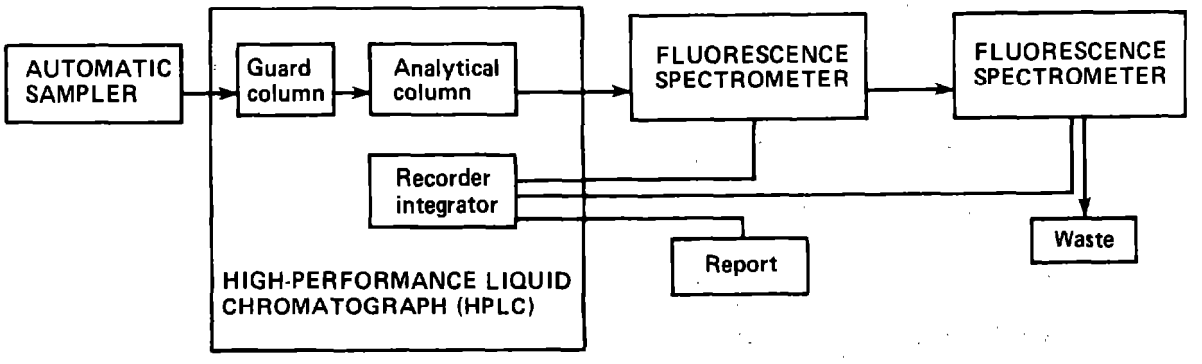
METHODS

A. Setup of Instruments Note: Flow diagram and timetable must be adjusted for the user's particular instruments.

1. Flow Diagram



arrows = sample flow; lines = electrical connections.

2. Timetable

Time (min)	Instrument	Instruction
< 0	autosampler	Sample picked up; see 33 min below
0	HPLC	Gradient programming begins; signal sent to autosampler to inject sample.
0	autosampler	Sample injected; signal sent back to HPLC to start data system.
0-22	HPLC	Gradient programming; see "Gradient Elution" below.
7	HPLC	Data system begins integrating both channels.
22	HPLC	Data system ends integration of channel 2. (See B.3 below)
25-35	HPLC	Reequilibration of column.
28	HPLC	Data system ends integration of channel 1. Report is printed.
33	autosampler	New sample is picked up.
35	HPLC	Solvent programming finished; new analysis can begin.

B. Operating Parameters of Instruments - Note: All operating parameters pertain to our. instruments. Users should adjust parameters to fit their instruments.

1. Automatic sampler

run time = 33 min

sample size =5 uL

mode = automatic

2. HPLC

Oven temperature = 50°+ 1°C

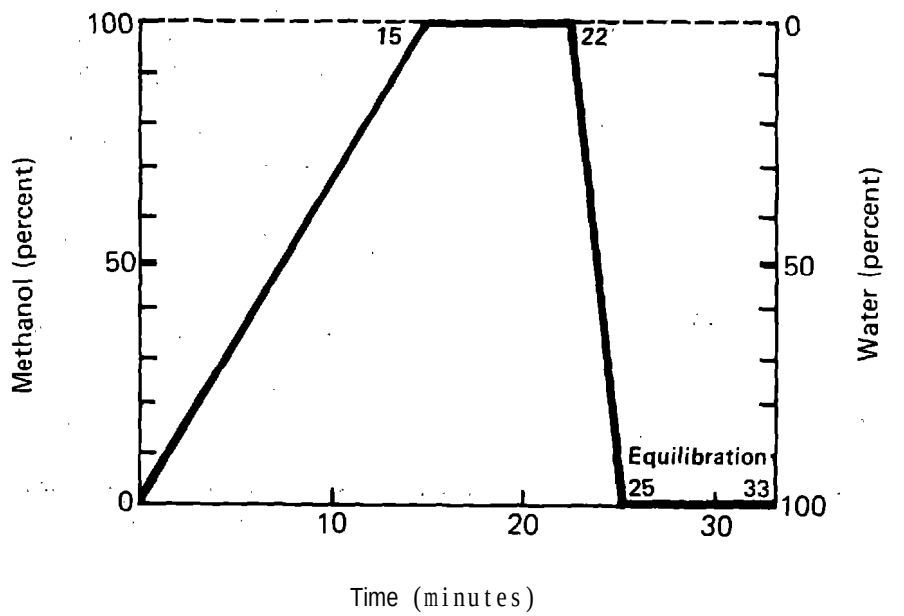
Data system: Run time = 28 min

Channel 1 = signal from the MPF 44A

Channel 2 = signal from the LS-4

Chart speed = 0.5 cm/min

Gradient elution



Instrument settings for linear gradient elution:

Water*(%)	Methanol.(%)	Flow (mL/min)
0	100	0	l.0
15	O	100	1.0
22	0	100	1.0
25	l00	0	1.5
33	100	0	1.0
35	100	0	1.0

* contains 5 uL of acetic acid/L of water

3. Fluorescence spectrometer(s)

MPF-44A: 380/430 nm excitation/emission for BaP wavelengths; integration from 7-28 min; see "Sample Analysis" on page 17.

slit width = 12 nm for both excitation and emission

response time = 0.3 set

sensitivity (gain) = 1

LS-4: 290/335 nm excitation/emission for NPH wavelengths; integration from 7-22 min; see "Sample Analysis" on page 17.

slit width = 10 nm for both excitation and emission

response time = 0.5 set

sensitivity (gain) = 0.25-l

Note: The wavelength pairs may be interchanged on the spectrometers (e.g., 380/430 nm may be selected on the LS-4 and 298/335 nm on the MPF-44A).

C. Sample Collection and Storage

1. Capture demersal fish by otter trawl.
2. After capture, place the fish in holding tanks containing fresh seawater until the fish can be necropsied aboard the research vessel.
3. Wash down all the dissection tools with methanol.
4. After sacrificing each fish, open the body cavity and excise the gall bladder.
5. Transfer the bile to a collection vial by holding the gall bladder above the vial and puncturing the gall bladder with the #ll scalpel blade.
6. Immediately place vials containing the bile samples on ice for transportation to the laboratory.
7. At the laboratory, transfer a 50 uL-portion of each sample to an autosampler vial, then store the two portions of each sample in a freezer at -2O°C.

D. Quality Assurance (QA)

1. Analyze at least two calibration standards at the beginning of each set of analyses. Two consecutive calibration runs must agree within a relative standard deviation (RSD) + 5% before proceeding to the next step.
2. Analyze the bile pool (Materials, Subsection D) and check to see that the calculated concentrations agree with the previous day's bile pool concentrations within + 10%. Over extended periods of time (6-12 mo) the RSD of the bile pool concentrations is + 20% (n > 20).

3. Program (or load) the automatic sampler so that every sixth analysis is a calibration standard. Also, for every six samples analyzed, repeat the analysis of at least one sample.

E. Sample Analysis - Note: Analyze controls and/or blanks to ensure that contaminants from reagents, previous analyses, etc. do not interfere with analyses.

1. After the instruments and operating parameters have been set up as described above (Methods; Subsections A and B), inject 5 uL of bile directly into the HPLC system using the autosampler.

2. Record HPLC/fluorescence detector response at BaP and NPH wavelength pairs on the dual channel recorder-integrator. Typical chromatograms of the bile pool at these wavelengths are shown in Figure 1, page 18; (Laboratories with only one fluorescence detector must rerun the sample to record the chromatogram at the second wavelength pair.)

3. Program the data system to integrate the areas of peaks eluting after 7 min (and before 22 min at NPH wavelengths, 290/335 nm) in the chromatograms. (Areas of compounds eluting before 7 min are excluded, because no known AH metabolite reference standards elute there. The peaks occurring at 22-28 min at 290/335 nm are attributable to contaminants in the solvents that are not removed by distillation.)

Note: The 7 and 22 min cutoffs are based on retention times in our chromatograms, and they must be adjusted for the user's instruments.

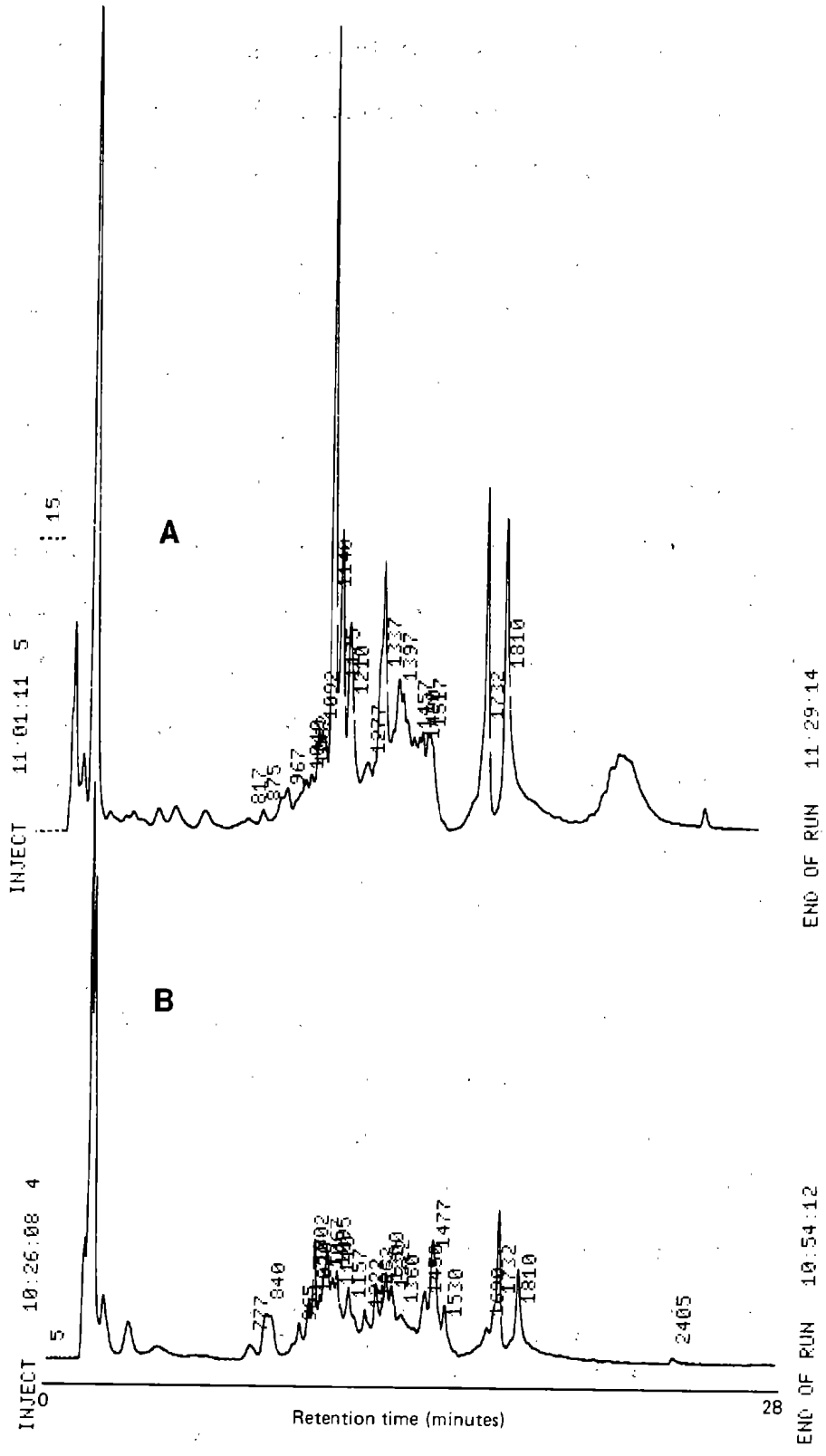


Figure 1. HPLC/fluorescence chromatograms of the "quality assurance bile pool" at (A) NPH wavelengths (290/335 nm, excitation/emission) and (B) BaP wavelengths (380/430 nm).

4. Calculate the density of bile by weighing a known volume of bile. Repeat this step four times, then calculate a mean density (n = 5).

F. Calculations

1. Identify the peaks from the BaP and naphthalene reference standards from the chromatograms of the calibration standard. Under our operating conditions, retention times are ca. 19 min for NPH and 24 min for BaP. Record the retention times and the integrated areas of each reference standard.
2. For each reference standard (BaP or NPH), calculate the mean integrated area for that day's runs. Report the mean + SD.
3. For each bile sample, sum the integrated areas of individual peaks, either automatically (using a data system) or manually. For two channel integrations, this results in "area sums" for both BaP and NPH.
4. Calculate the concentrations according to the following equations:

Eq. 1: Concentration of metabolites at BaP wavelengths (ng/g, wet wt) =

$$\left[\frac{\text{concentration of BaP}}{\text{mean area of BaP}}\right]_{\text{std}} \times \left[\frac{\text{area sum (7-28 min)}}{\text{density of bile}}\right]_{\text{sample}} \times \left[\frac{\mu\text{L std}}{\mu\text{L sample}}\right]_{\text{injected}}$$

Eq. 2: Concentration of metabolites at NPH wavelengths (ng/g, wet wt) =

$$\left[\frac{\text{concentration of NPH}}{\text{mean area of NPH}}\right]_{\text{std}} \times \left[\frac{\text{area sum (7-22 min)}}{\text{density of bile}}\right]_{\text{sample}} \times \left[\frac{\mu\text{L std}}{\mu\text{L sample}}\right]_{\text{injected}}$$

5. Report the results to 2 significant figures.

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ACKNOWLEDGMENTS

The analytical methods in this publication result from 7 years of research, development, application and revision by chemists at the National Analytical Facility. NAF has received extensive support from numerous organizations and individuals.

The interagency program between NOAA's National Ocean Service and Minerals Management Service, known as the Outer Continental Shelf Environmental Assessment Program, has funded the research from its inception. In addition, the National Status and Trends Program has provided an opportunity for extensive testing of the methods developed in our research.

Many individuals have contributed greatly to our work, We thank Drs. Donald C. Malins and Sin-Lam Chan; and Donald W. Brown, Victor Henry, Linda D. Rhodes, Mark S. Myers, Tracy K. Collier, Jim C. Drury, J. Richard Hughes, Douglas D. Weber, and William Gronlund.

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APPENDIX

NAF's Research on Metabolites of Aromatic Compounds in Fish

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