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PRESERVATION OF ORGANICS. PART III
STABILITY OF CHLOROPHENOLS IN PRESERVED FISH HOMOGENATES

by

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MANAGEMENT PERSPECTIVE

Concerns about chlorophenol pollution in the Fraser River Estuary in British Columbia has resulted in the monitoring of water, sediment and fish samples for phenols. Routine analysis of fish samples is particularly important since the esturary also supports one of the largest commercial fisheries in Canada. In response to a request generated by Water Quality Branch, Pacific and Yukon Region, a Study on the Preservation and Stability of Chlorophenols in Fish Homogenates was carried out. Results indicated that the integrity of chlorophenols in fish was maintained for a minimum of 15 weeks if the samples were kept frozen at -20°C.

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PERSPECTIVE - GESTION

Les préoccupations au sujet de la pollution par les chlorophénols dans l'estuaire du Fraser en Colombie-Britannique font suite à l'analyse des phénols dans les échantillons d'eau, de sédiments et de poissons. L'analyse courante des échantillons de poissons est particulièrement importante étant donné que l'estuaire supporte également l'une des plus grandes pêcheries commerciales au Canada. En réponse à une demande du bureau de la région du Pacifique et du Yukon de la Direction de la qualité de l'eau, une Étude sur la préservation et la stabilité des chlorophénols dans les homogénéisats de poisson a été faite. Les résultats indiquent que l'intégrité des chlorophénols contenus dans les poissons sont maintenus pendant une période minimale de 15 semaines lorsque les échantillons sont congelés à -20°C.

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ABSTRACT

This report summarizes the results of a 2-part study for the preservation of chlorophenols in fish. Homogenates of a clean, laboratory-grown lake trout were individually spiked with a mixture of 20 chlorophenols so that the fortification level was 200 ng/g for each phenol. Another homogenate was prepared from a composite sample of 60 small naturally contaminated starry flounders collected from Fraser River, B.C. This sample was later shown to be contaminated by four native chlorophenols at levels below 20 ng/g. Subsamples of these two homogenates were then stored at -20°C in the dark for up to 15 weeks. Replicate samples were removed for analysis at 0-time, and 3, 6, 10 and 15 weeks after storage. The analysis of chlorophenols in fish involved soxhlet extraction, removal of fish lipids by GPC, back extraction of chlorophenols into K_2CO_3 solution, acetylation of phenols with acetic anhydride, silica gel column cleanup of the acetate derivatives, as well as GC-ECD and GC-MSD analysis of the final extracts. In both cases, analytical results indicated no degradation of any chlorophenol in the preserved fish samples. Therefore, the integrity of chlorophenols in fish can be maintained for a minimum of 15 weeks if the samples are kept at -20°C in the dark.

RÉSUMÉ

Le présent rapport résume les résultats d'une étude à deux volets portant sur la préservation des chlorophénols dans les poissons. Des homogénéisats de touladis propres élevés en laboratoire ont été enrichis individuellement d'un mélange de 20 chlorophénols de façon à ce que le niveau de fortification soit de 200 ng/g pour chaque phénol. Un autre homogénéisat a été préparé à partir d'un échantillon composite de 60 petites plies étoilées contaminées en milieu naturel capturées dans le Fraser (C.-B.). Après analyse, on a trouvé que cet échantillon était contaminé par quatre chlorophénols naturels à des teneurs de 20 ng/g. Des sous-échantillons de ces deux homogénéisats ont ensuite été entreposés à -20°C, à la noirceur, pendant 15 semaines. Des exemplaires d'échantillons ont été retirés pour analyse au moment zéro, et après 3, 6, 10 et semaines d'entreposage. L'analyse des chlorophénols dans les poissons comprenait les étapes suivantes : extraction au soxhlet, élimination des lipides par chromatographie sur gel, extraction inverse des chlorophénols dans une solution de K_2CO_3, acétylation des phénols avec de l'anhydride acétique, nettoyage des dérivés d'acétate dans une colonne de Silicagel et analyse CG-DCE et CG-SM des extraits finals. Dans les deux cas, les résultats analytiques n'ont indiqué aucune dégradation d'aucun des chlorophénols présents dans les échantillons de poisson conservés. On peut donc préserver l'intégrité des chlorophénols contenus dans le poisson pendant au moins 15 semaines, à condition que les échantillons soient gardés à la noirceur, à -20°C.

1.0 INTRODUCTION

Pentachlorophenol and a few other chlorophenols are used to preserve wood by the lumber industries. Since large amounts of phenols are in constant use every year, residues of these compounds create a serious contamination problem to the environment in the areas where wood preserving activities are heavy. In previous studies (1,2), detectable amounts of chlorophenols were found in water, sediment and biota samples collected from the Fraser River Estuary, thus routine monitoring of such samples for chlorophenols is necessary in order to determine the water quality and to provide early warning if high levels of chlorophenols are found. The analysis of fish samples is especially important since the Fraser River Estuary supports one of the largest commercial fisheries in North America.

Due to the centralization of the Water Quality laboratories, samples from across Canada have to be shipped to Burlington and possibly stored for some time before being analyzed. Therefore, it is necessary to evaluate the preservation and stability of the analytes during transportation and storage period. In Part II of the preservation studies, we have reported the stability of 20 chlorophenols in fortified natural water samples over a period of 15 weeks. The present study evaluates the preservation and stability of 20 chlorophenols in both fortified and naturally contaminated fish homogenates.

2.0 STUDY DESIGN

In the past few years, we have developed several methods for chlorophenols analysis by the formation of their acetate (3,4), chloroacetate (5) and pentafluorobenzyl ether (6) derivatives. For reasons of simplicity in derivatization, ruggedness and interference, the acetate method was used in this work. In order to cover the entire range of chlorophenols, the following 20 were included in the

present study: 2-chlorophenol (MCP), 3-MCP, 4-MCP, 2-chloro-5-methylphenol, 4-chloro-3-methylphenol, 2,6-dichlorophenol (DCP), 2,4-DCP, 3,5-DCP, 2,3-DCP, 3,4-DCP, 2,4,6-trichlorophenol (TCP), 2,3,6-TCP, 2,3,5-TCP, 2,4,5-TCP, 2,3,4-TCP, 3,4,5-TCP, 2,3,5,6-tetrachlorophenol (TECP), 2,3,4,6-TECP, 2,3,4,5-TECP and pentachlorophenol (PCP). A mass selective detector was used to determine the monochlorophenols and chloromethylphenols since their acetate derivatives were not sensitive to electron-capture detection. The rest of chlorophenols were determined by electron-capture detector.

The preservation study was done in two parts. Part one involved the homogenization, spiking, preservation and analysis of a laboratory grown trout which was free from chlorophenol contamination. Since a mixture of the above 20 chlorophenols was used to spike the fish, information regarding the stability of all common chlorophenols can be obtained in this part of the study. In part two, a composite homogenate of 50 to 60 starry flounders collected in various parts of the Fraser River was prepared and used.

Starry flounders were chosen for the preservation study because they are a popular dietary fish and are also bottom dwellers so that their bodies are most likely to accumulate toxic organics from the sediments. The areas of collection for the starry flounders (Table 1) were selected by Water Quality Branch, Pacific and Yukon region so that the samples were most representative for the Fraser River Estuary. Since a previous report (1) has indicated that the same type of fish collected in that areas was naturally contaminated with chlorophenols, no spiking was done for the composite sample. In this case, the results would provide the most realistic assessment of the stability of chlorophenols in fish tissues since the toxins were naturally accumulated in the samples.

A study period of 15 weeks was arbitrarily chosen and replicate samples were analyzed immediately after preparation, and three, six, ten and fifteen weeks after storage. No chemical

preservative was added to the fish tissues. All subsamples were sealed and preserved by storage at -20°C in the dark.

A total of over 40 fish subsamples were analyzed for this preservation study. The tedious analytical procedure and the limited resources allocated did not permit the inclusion of more than two types of fish in this study.

3.0 EXPERIMENTAL

3.1 Standards and Reagents

All chlorophenol standards were obtained from Aldrich Chemical Co. except that a recrystallized sample of 2,3,4,6-tetrachlorophenol was obtained from Dr. J. Carey (Lakes Research Branch, NWRI). Individual chlorophenol stock solutions of 1000 ppm were prepared. Acetic anhydride was purchased from BDH and was triple-distilled before use. Phenanthrene-d_{10} internal standard for mass selective detection was obtained from USEPA. For the other reagents, see Ref. 3. A mixture of the 20 chlorophenols in acetone was prepared for spiking and the preparation of calibration standards.

3.2 Homogenization, Subsampling, Fortification and Preservation

A laboratory grown lake trout of about 1 kg in body weight was used to prepare the fortified samples. The body tissues of the trout were homogenized in an Oysterizer blender and 5g aliquots of the homogenate were subsampled into small jars with the sample weight recorded to the nearest 0.01 g. Each smple was then spiked with 200 µL of the above chlorophenol mixture (5 µg/mL) so that the solution was evenly distributed to the entire tissue sample. The jars were covered with a piece of solvent washed aluminum foil and sealed tightly with a cap. All samples were then kept at -20°C in the dark until analysis.

About 50 to 60 starry flounders with a total weight of 500 g were also blended to yield a composite whole fish homogenate.

Subsamples of 5 g each were also prepared and preserved as for the trout sample.

3.3 Analytical Procedure

After storage time had elapsed, the entire fish homogenate in each bottle was thawed and quantitatively transferred to a mortar and mixed with 10.0 g of anhydrous sodium sulfate. The mixture was then placed on top of 5 cm of Celite 545 in a sintered-glass thimble and extracted in a Soxhlet apparatus with 300 mL of 41/59 hexane/acetone mixture for 8 hrs. at a rate of 10 to 12 cycles per hour.

The organic extract was evaporated down to ca. 5 mL using a 3-stage Snyder column and the concentracted extract was made up to either 25 mL (for the trout) or 10 mL (for the starry founders) with 1/1 dichloromethane/cyclohexane. A 5.0 mL aliquot of this extract was injected onto a 60g Bio-Beads S-X3 GPC column and eluted with 1/1 dichloromethane/cyclohexane mixture at a constant flow rate of 5.0 mL/min. The first 145 mL of the eluant containing most of the lipids were discarded. The next 165 mL of eluant was collected, evaporated down to ca. 80 mL and made up to 100 mL with cyclohexane. The phenols in the second fraction were then back-extracted into 1% K_2CO_3 in three consecutive extractions using 40, 30 and 30 mL of the base.

An extractive acetylation of the phenols was carried out by stirring the K_2CO_3 solution with 2 mL of acetic anhydride and 25 mL of petroleum ether for 30 min. This process was repeated twice and the combined petroleum ether layer was dried with anhydrous sodium sulfate and evaporated down to ca. 5 mL with a Snyder column in the presence of 2 mL of isooctane as a keeper. The petroleum ether layer was further evaporated to 2 mL using a gentle stream of nitrogen. A 5.0 cm 5% deactivaed silica gel column was prepared with a 23 cm long disposable Pasteur pipet. The acetylation products were applied to the silica gel column and the acetates of chlorophenols were collected by eluting the column with 10 mL of benzene. The benzene-fraction was

then evaporated and solvent exchanged to a final volume of 2.0 mL with toluene. For the starry flounder samples, the benzene fraction was further evaporated to 0.5 mL because of low chlorphenol levels. For each set of samples, an aliquot of the chlorophenol spiking solution was also derivatized, cleaned up and used as an external standard for instrument calibration.

For GC-ECD analysis, a 25 m x 0.25 mm i.d. OV-1 capillary column and the following temperature program was used: initial column temperature, 70°C, initial time, 0.5 min. oven temperature programming rate 1, 10°C/min (70° to 130°C), held at 130°C for 5 min, programming rate 2, 2°C/min (130° to 170°C), held at 170°C for 5 min. Carrier gas was helium and linear velocity was 25 cm/sec. A two uL aliquot was injected in the splitless mode by an autosampler with the splitless valve on for 0.5 min.

For the analysis of monochlorophenol and chloromethylphenol acetate derivatives, a GC-MSD system was used. In this case, a 30 m x 0.25 mm i.d. SPB-5 capillary column and the following temperature program was used: column initial temperature, 70°C, initial time, 0.5 min, programming rate 1, 30°C/min. (70° to 120°C), held at 120°C for 5 min., programming rate 2, 5°C/min. (120° to 170°C), held at 170°C for 15 min. Before MSD analysis an aliquot (20 µL) of a 100 ppm phenanthrene-d_{10} solution was added to the extract and calibration standard as an internal standard to calibrate MSD responses. For quantitative purposes, the MSD was operated in the selected ion monitoring mode and the molecular ions of the monochloro- and chloromethyl- phenols were monitored.

4.0 RESULTS AND DISCUSSION

Recoveries of the 20 chlorophenols from the fortified fish samples after storage for various periods of time are tabulated in Table 2. A fish tissue before fortification was also analyzed and showed no detectable amounts of chlorophenols in the blank. The average recoveries of chlorophenols in the preserved samples were

between 85 and 105% over the 15-week study period and the relative standard deviations of replicate anlysis were below 12% in most cases. Although the precision of analysis was slightly worse than those for water and sediment samples, the variation in results could mostly be attributed to the tedious procedures for the analysis of chlorophenols in fish tissues. There was not a single case that a continuous decline in recovery was observed to suggest degradation of any chlorophenol in the preserved fish samples.

Only a few chlorophenols, namely, PCP, 2,3,4,6-TECP, 2,3,4,5-TECP and 2,4,6-TCP were found in the composite starry flounder sample. The presence of such phenols was in agreement with the findings for similar types of fish in the Fraser River Estuary reported earlier (1,7). Because the levels of these native phenols were low in the composite sample (20 ppb or lower), the final extracts were further concentrated down to 0.5 mL before GC-ECD analysis was performed. As indicated in Table 3, no decline in the recovery of the four chlorophenols was observed during the study and therefore no degradation of the phenols in the naturally contaminated fish could be suggested.

It should be noted that the starry flounders were collected approximately three weeks before the composite sample was prepared (see Table 1) and the samples were kept frozen during storage and transportation. Therefore, when the composite subsamples were analyzed, the true storage time was actually about three weeks longer than the nominal storage time. Due to the breakdown of our freezer near the end of the study (14th week), the starry flounder samples were thawed to ambient temperature for about 12 hours before they were transferred to another -20°C freezer. Although the fish tissues became more rubbery in texture and the lipids were partially separated, the analytical results did not indicate any degradation of chlorophenols in the starry flounders due to this incidence.

The results derived from this 2-part study using both fortified and naturally contaminated fish homogenates indicated that

chlorophenols were stable over a 15-week study period when the samples were preserved under deep-freeze (-20°C) conditons.

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TABLE 1

Starry flounder samples collected from Fraser River, B.C. and used in this study

Sample No.	Collection Date	Collection Site	Approximate Weight
870058	4/27/87	North arm of Fraser River, west tip of Mitchell Island	20 g
870059	4/27/87	North arm of Fraser River, northwest shore of Mitchell Island	40 g
870060	4/27/87	North arm of Fraser River, northshore, across from Mitchell Island	10 g
870062	4/27/87	North arm of Fraser River, north shore of Mitchell Island	50 g
870063 870064	4/30/87	North arm of Fraser River, north shore across from Richmond Island	180 g
870065	4/30/87	North arm of Fraser River, south shore, across from Marine Drive Golf Course	200 g

TABLE 2

Mean % recoveries of chlorophenols in preserved, fortified fish homogenates (n = 4)

PHENOL	Week 0		Week 3		Week 6		Week 10		Week 15	
	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
2-Chloro	90.6	3.3	91.2	10.7	87.4	7.6	90.6	8.3	99.0	4.9
3-Chloro	98.6	3.4	102.5	12.5	100.2	8.8	103.8	6.9	105.3	2.5
4-Chloro	98.3	5.1	100.0	11.8	103.3	12.9	102.9	7.8	99.9	7.3
2-Cl-5-Me	94.8	4.1	94.6	12.5	90.3	7.8	95.3	8.1	100.8	10.7
26-Dichloro	94.5	8.9	84.9	7.4	93.3	7.1	92.8	7.3	100.9	7.6
4-Cl-3-Me	95.9	7.0	98.0	13.2	95.6	7.0	103.6	7.5	111.0	4.1
24-Dichloro	100.6	11.4	90.3	5.7	92.8	7.5	102.3	6.9	94.2	9.1
35-Dichloro	101.1	10.3	91.9	5.4	92.6	7.5	99.2	4.6	100.3	8.8
23-Dichloro	108.8	10.0	95.3	6.8	98.4	8.2	103.0	4.4	94.7	8.6
34-Dichloro	103.4	10.7	88.7	10.6	96.5	7.2	104.2	5.6	104.0	9.8
246-Trichloro	88.5	14.6	80.5	10.8	93.2	8.5	94.8	7.5	94.7	10.2
236-Trichloro	101.5	15.0	90.4	8.8	91.2	5.7	97.3	7.3	101.4	10.2
235-Trichloro	96.5	11.0	84.9	11.4	100.8	4.6	96.1	7.2	103.1	11.2
245-Trichloro	102.0	11.2	84.5	10.7	108.0	6.8	101.6	7.5	102.2	10.1
234-Trichloro	98.2	13.4	85.6	10.3	93.9	7.1	96.3	9.5	108.8	13.8
345-Trichloro	99.6	15.1	87.3	8.3	93.6	7.2	97.7	9.2	105.7	14.1
2356-Tetrachloro	104.1	18.0	94.1	10.5	103.8	9.2	93.5	7.0	96.4	11.4
2346-Tetrachloro	106.3	18.2	96.2	12.3	114.5	8.8	101.5	8.8	91.2	10.0
2345-Tetrachloro	94.9	13.1	83.5	9.6	88.2	7.9	96.1	11.0	112.2	8.9
PCP	102.6	12.3	92.5	10.6	92.7	7.6	102.6	7.9	100.3	12.3

TABLE 3

Chlorophenol levels (ng/g) in preserved starry flounder homogenates
(mean ± s.d., replicate = 4)

PHENOL	Week 0	Week 3	Week 6	Week 10	Week 15
2,4,6-Trichloro	2.3 ± 0.5	2.1 ± 0.1 (92.3%)*	2.5 ± 0.5 (108.7%)	2.5 ± 0.2 (108.7%)	2.2 ± 0.5 (95.7%)
2,3,4,6-Tetrachloro	17.3 ± 3.5	17.8 ± 2.6 (102.9%)	18.0 ± 2.8 (104.0%)	17.6 ± 1.7 (101.7%)	18.4 ± 2.6 (106.4%)
2,3,4,5-Tetrachloro	0.9 ± 0.3	1.1 ± 0.2 (122.2%)	1.2 ± 0.2 (133.3%)	1.4 ± 0.1 (155.6%)	0.9 ± 0.1 (100.0%)
PCP	20.6 ± 2.6	20.2 ± 1.9 (98.1%)	20.7 ± 4.2 (100.5%)	19.9 ± 1.7 (96.6%)	21.3 ± 0.4 (103.4%)

* Relative to week 0.