

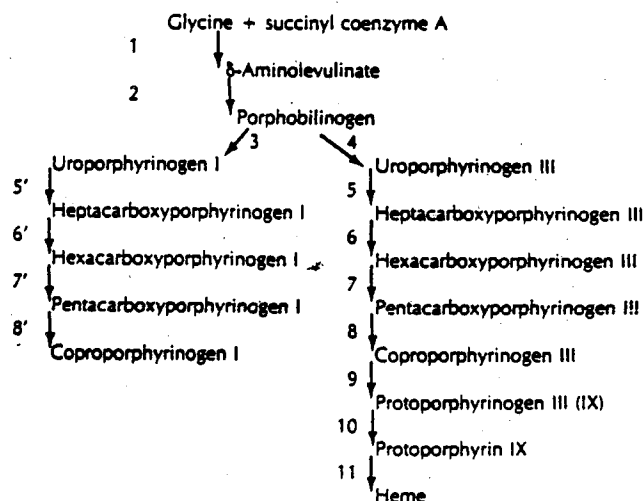
Laboratory Report

Porphyrinogens, Porphyrins, and the Porphyrrias

The porphyrias are consequences of impairment either in the formation of the porphyrinogens from porphobilinogen or in the transformation of the porphyrinogens to heme.¹ Such an impairment causes a pathologic accumulation of porphobilinogen or porphyrins (or both) in tissues and body fluids.

The manifestations of the porphyrias include photosensitivity of the skin, persistent abdominal aching or pain, and neuropathy with widely variable expression that may affect the central, autonomic, and peripheral systems and include mental disturbance, motor dysfunction, and sensory loss. The cutaneous photosensitivity may range from very mild to very severe with mutilating lesions. The neuropathy of porphyria is especially significant in that it can progress to life-threatening respiratory paralysis (Table 1).

The formation of the porphyrinogens and heme from glycine and succinyl coenzyme A occurs in human cells of nearly all types and includes the following 15 major enzyme-catalyzed steps:



Only the porphyrinogens of the series III are precursors of heme, and the isomeric porphyrinogens of the series I and the porphyrins of both series I and III are by-products with no known physiologic functions; the porphyrins are formed from the porphyrinogens by nonenzymic oxidation.

As shown in Table 1, seven forms of porphyria are known to occur, and with one exception, each form is inheritable and is caused by or potentiated by an abnormally low activity of one of the several enzymes involved in the formation of the porphyrinogens and heme.

Two forms of porphyria are expressed primarily in the erythropoietic system. Congenital erythropoietic porphyria is a rare disorder that is readily recognizable in neonatal life by the voiding of dark or wine-red urine; uroporphyrin I is primarily responsible for the color of the urine. Congenital erythropoietic porphyria is characterized also by moderate to extreme photosensitivity, hypertrichosis, hemolysis, splenomegaly, and increased uroporphyrin and coproporphyrin in the erythrocytes and blood plasma. Examination of the bone marrow in violet light (400 to 410 nm) shows red fluorescence of excessive porphyrins in the cytoplasm of red cell precursors. In addition, in most known cases uroporphyrin is deposited in the teeth and can be demonstrated by red fluorescence when exposed to violet light. Protoporphyria is relatively mild and is expressed in both the erythropoietic system and the liver. It is characterized by photosensitivity resulting in acute solar urticaria and chronic solar eczema; the cutaneous lesions usually heal in hours or days without scarring. The chemical features include increased erythrocyte free protoporphyrin and increased fecal excretion of protoporphyrin. Urinary porphyrins and porphyrin precursors in most cases of protoporphyria have not been increased.

A third and very rare form of erythropoietic porphyria has clinical characteristics like those of congenital erythropoietic porphyria and is expressed chemically by increased protoporphyrin in erythrocytes, increased urinary excretion of uroporphyrin III, heptacarboxyl porphyrin III, and an unidentified porphyrin, and increased fecal excretion of coproporphyrin, tricarboxyl porphyrin, and protoporphyrin.²

A fourth form of erythropoietic porphyria has been observed but has not been characterized fully in chemical terms. In one reported case³ and in one case of a neonate evaluated in the Mayo Medical Laboratories, extreme photosensitivity and porphyrinuria were observed, and erythrocytes

Table 1.—Acknowledged Forms of Porphyrin

Condition	Mode of inheritance	Enzyme deficient*	Clinical expression
Erythropoietic porphyrias			
Congenital erythropoietic porphyria	Autosomal recessive	Uroporphyrinogen III cosynthase (step 4)	Photosensitivity, hemolytic anemia, hypertrichosis
Protoporphyria	Autosomal recessive	Ferrochelatase (step 11)	Photosensitivity
Hepatic porphyrias			
Acute intermittent porphyria	Autosomal dominant	Uroporphyrinogen I synthase (step 3)	Neuropathy, abdominal pain, psychosis
Hereditary coproporphyria	Autosomal dominant	Coproporphyrinogen oxidase (step 9)	Same as acute intermittent porphyria
Variegate porphyria	Autosomal dominant	Protoporphyrinogen oxidase or ferrochelatase (step 10 or 11)	Same as acute intermittent porphyria + photosensitivity
Porphyria cutanea tarda	?	Uroporphyrinogen decarboxylase (steps 5-8)	Photosensitivity
Intoxication porphyria	Acquired	Variable	Abdominal pain, neuropathy

*Numbered steps refer to the steps in the described reaction sequence for the formation of porphyrinogens.

Table 2.—Some Substances Reported to Induce Attacks of Acute Intermittent Porphyrin¹

Barbiturates
Sulfonamides
Phenytoin (Dilantin)
Meprobamates (Miltown and Soma)
Griseofulvin
Methsuximide
Dichloralphenazone
Pyrazolones (antipyrenes)
Methprylone
Imipramine
Eucalyptol
Ergot substances
Chlordiazepoxide (Librium)
Estrogens
Contraceptive steroids

contained increased coproporphyrin and normal concentrations of protoporphyrin.

Acute intermittent porphyria, coproporphyria, variegate porphyria, and porphyria cutanea tarda have been classified as "hepatic porphyrias" because it is assumed that errors in porphyrinogen synthesis are predominantly expressed in hepatocytes. Acute intermittent porphyria, coproporphyria, and variegate porphyria are characterized by acute attacks of abdominal pain and by hypertension, neuropathy, and mental disturbance that may last from several days to several months. Chronic photosensitivity and sensitivity of the skin to mechanical trauma are usual characteristics of variegate porphyria but not of acute intermittent porphyria; in coproporphyria, sensitivity of the skin to light and to mechanical trauma may be pronounced, mild, or nonexistent. In readily measurable chemical terms, acute intermittent

porphyria is characterized by an increased urinary excretion of porphobilinogen during acute attacks and by low activity of uroporphyrinogen I synthase in erythrocytes. Coproporphyria may be expressed in part by increased coproporphyrin in erythrocytes and by increased urinary excretion of coproporphyrin; however, the pathognomonic finding is persistently increased fecal excretion of coproporphyrin III. Similarly, variegate porphyria may cause increased urinary excretion of coproporphyrin, but the distinguishing characteristic is increased fecal excretion of coproporphyrin and protoporphyrin. In addition, variegate porphyria may cause increases in the concentrations of porphyrins in the blood plasma. Acute attacks of acute intermittent porphyria, coproporphyria, and variegate porphyria may be provoked by medications, and a variety of other commonplace substances are suspect. Table 2⁴ lists some of the medications reported to induce attacks. The sensitivity of affected persons to barbiturates is especially noteworthy. The use of pentothal as an anesthetic during surgery or during dental procedures would be especially risky, and the use of barbiturates to control psychiatric disturbances could cause exacerbations of the symptoms.

Porphyria cutanea tarda is characterized by photosensitivity and increased urinary excretion of uroporphyrinogen I, uroporphyrin I, and the heptacarboxyl porphyrinogen and porphyrin of series III. Sensory neuropathy may occur in association with this disorder, but episodic abdominal pain, motor dysfunction, and mental disturbance are not characteristics. Porphyria cutanea tarda appears to be potentiated by a partial deficiency of

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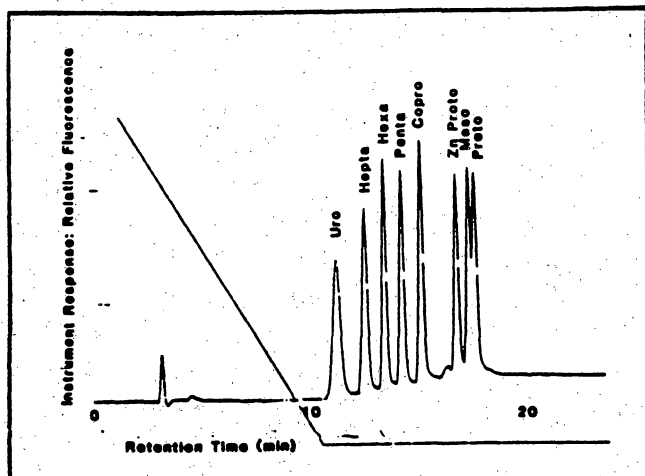


Fig. 1. Chromatogram of porphyrins standard. Injected sample consisted of 5 pmol of each standard porphyrin: octacarboxyl (Uro), heptacarboxyl (Hepta), hexacarboxyl (Hexa), pentacarboxyl (Penta), tetracarboxyl (Copro), and dicarboxyl (Meso, Proto, and Zn Proto) porphyrins.

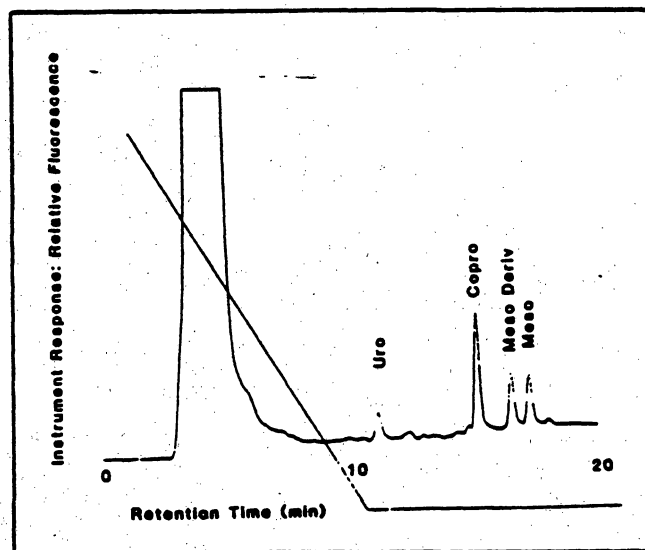


Fig. 2. Porphyrins in urine from a healthy nonporphyric male. Injected sample volume, 100 μ l. Abbreviations are as in Figure 1.

uroporphyrinogen decarboxylase, and its clinical expression is provoked by factors that include iron overload,⁵ chronic abuse of alcohol, and prolonged use of estrogens or contraceptive steroids. A porphyria cutanea tarda-like disorder has been caused in at least two cases by porphyrin-producing hepatic adenomas;⁶ in one of those cases, the porphyria disappeared after removal of the tumor. In addition, postmortem

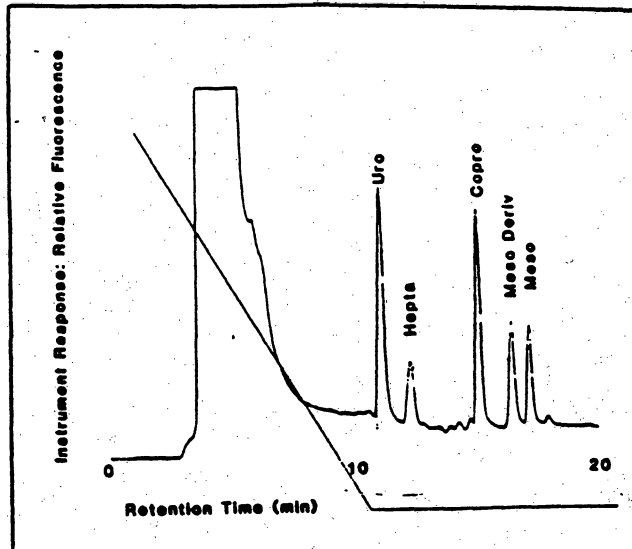


Fig. 3. Porphyrins in urine from a male patient with porphyria cutanea tarda. Injected sample volume, 200 μ l. Abbreviations are as in Figure 1.

examinations of 36 persons with porphyria cutanea tarda revealed hepatocellular carcinomas in 17 cases (47%);⁷ the significance of that observation is uncertain. Early signs of porphyria cutanea tarda include enhanced facial pigmentation and hypertrichosis of the forehead, malar region, and forearms.

Intoxication porphyria is characterized by increased erythrocyte protoporphyrin (especially the zinc-complexed form), increased urinary excretion of δ -aminolevulinate and porphobilinogen, and increased fecal excretion of protoporphyrin; the urinary excretion of porphyrinogens and porphyrins can be increased also. This condition can be caused by ingestion of or exposure to any of a variety of toxic substances; probably most of the recognized cases have been caused by ingestion of lead, hexachlorobenzene, or 2,3,7,8-tetrachlorodibenzo-p-dioxin. Heavy metals, halogenated aromatic hydrocarbons, and a variety of other chemicals can cause suppression of enzymes involved in porphyrinogen metabolism, with an accumulation of intermediates. Enzymes known to be suppressible by toxic substances include δ -aminolevulinate dehydratase, uroporphyrinogen I synthase, and ferrochelatase. Probably other porphyrinogen-related enzymes, also, are susceptible.

In the past, the separate quantitation of uroporphyrins, coproporphyrins, and protoporphyrins in patients' specimens involved tedious procedures for

Table 3.—Reference Values⁷⁻⁹

Blood (µg porphyrins/dl)	Protoporphyrin		Other porphyrins		
Erythrocytes					
Men (n = 39)	17-52		<1		
Women (n = 50)	16-65		<1		
Plasma					
Men and women	<1		<1		
Urine	Uro-porphyrin		Hepta-carboxylic	Hexa-carboxylic	Penta-carboxylic
					Copro-porphyrin
(µg porphyrins/24 h)					
Men (n = 33)					
Median	16		7	3	2
Mean (SD)	20 (11)		7 (3)	3 (1)	2 (1)
Range	8-44		0-12	0-5	0-4
Women (n = 24)					
Median	11		4	2	1
Mean (SD)	11 (5)		5 (2)	2 (1)	1 (1)
Range	4-22		3-9	0-5	0-3
Men and women (n = 57)					
Median	13		6	2	1
Mean (SD)	17 (10)		6 (3)	2 (1)	1 (1)
Range	4-44		0-12	0-5	0-4
Porphyria cutanea tarda (n = 15)					
Range	104-5,177		43-1,508	0-161	0-305
					7-263
Feces (µg porphyrins/24 h)	Uroporphyrins		Coproporphyrins		Protoporphyrins
	<1,000		<200		<1,500

differential extractions. Recently, the quantitative analysis has been improved greatly in terms of procedural convenience, accuracy, and specificity by the development of methods for simplified extractions of the total porphyrins and by applications of high-performance liquid chromatography to the analysis of buffered urine directly and of extracts of blood.⁸⁻¹⁰ Figures 1 to 3 illustrate some features of porphyrins analysis by high-performance liquid chromatography in our laboratories. Figure 1 presents a chromatogram of the standard mixture of purified porphyrins used for calibration of the chromatography system and illustrates the separational power and sensitivity of the system. The system is calibrated in terms of the size of

the instrument response (peak size on the chart) for a measured quantity of each porphyrin in the standard mixture. Quantities of the porphyrins in the patients' specimens are determined from the sizes of the porphyrin peaks in the charts (Fig. 2 and 3) and the instrument response data from the primary standard mixture. The test data are converted to and reported as quantities of porphyrins excreted per 24 hours, calculated by means of the volumes of the samples analyzed (usually 100 to 200 µl) and the total volumes of the timed collections. The various porphyrins appear in the eluate at different times and are identified, therefore, by the respective retention times (elution times). In cases in which the presence

Table 4.—Recommended Tests

Suspected porphyria	Recommended tests
Congenital erythropoietic porphyria	Urinary porphyrins
Protoporphyria	Urinary, fecal, erythrocytic porphyrins
Acute intermittent porphyria	Urinary porphobilinogen, porphyrins; erythrocyte uroporphyrinogen I synthase
Hereditary coproporphyria	Urinary porphobilinogen, porphyrins; fecal porphyrins; erythrocyte uroporphyrinogen I synthase
Variegate porphyria	Urinary porphobilinogen, porphyrins; fecal porphyrins; erythrocyte uroporphyrinogen I synthase
Porphyria cutanea tarda	Urinary porphyrins
Intoxication porphyria	Erythrocytic porphyrins; urinary δ-aminolevulinate, porphobilinogen, porphyrins; fecal porphyrins

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or quantity of porphyrin in a chromatographic fraction (peak) is doubtful, the fraction is trapped in a collector and evaluated by scanning spectrofluorometry. In the figures, the intervals (minutes) required for elution of the porphyrins are shown on the abscissas.

Uroporphyrin is shown to elute from the chromatographic column at 10 to 11 minutes after injection of the specimen sample, whereas elution of coproporphyrin requires 15 to 16 minutes and elution of protoporphyrin requires 18 to 19 minutes. Figure 3 presents an example of an analysis of a porphyric urine—in this case a porphyrins profile typical of porphyria cutanea tarda—showing large increases of uroporphyrin and heptacarboxyl porphyrin; those features are readily evident on comparison with the normal profile in Figure 2. The mesoporphyrin (a dicarboxylic porphyrin) and the mesoporphyrin derivative shown in the chromatograms were added to the specimens for use as internal standards; dicarboxylic porphyrins are usually not excreted in the urine.

Reference values for porphyrins in blood, urine, and feces are given in Table 3.^{8,9,11}

Table 4 is offered as a guide to the selection of tests to establish the diagnosis of porphyria and to identify the specific form for any individual case.

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Section of Clinical Chemistry,
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Nº 1832140

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TCDD

JOB NO.

2,4-Dichlorodibenzo-p-dioxin

PREPARED BY (SIGNATURE)

Fred Williams

DATE

27 Jan

320.0100

Four samples of 2,4 Dichlorodibenzo were analyzed for TCDD Disposed. The samples were KL 81-7001 and KR 12-7069 (from G Baurtegin) and KM 01-7003 and KM 07-7032 (from previous Runmick work). Sample 7003 had been found to contain ≈ 9.5 ppb of TCDD's colution. Batch 2,3,7,8-TCDD. Sample 7032 was found to be free of TCDD's at 0.9 ppb.

Samples were analyzed by T. Mayer on the HP 5985B GC/MS using label 10C columns column under conditions described by Paul et al. Used Chem 52, 1980 pop 2257 to 2263. Samples were prepared using 20% KOH and conc. H_2SO_4 washed to limit impurities as much as possible.

FEID	Sample Name	Wt.	322	334	TCI
9529	1st R-1 + 1st Toluene	-	1316	512	-
9530	IS-1 7032 2nd/200	5.08	-	2924	101
9531	IS-2 7001 2nd/200	3.22	165	2127	7
9532	IS-3 7001 2nd/200	3.39	126	2338	5
9533	IS-4 7069 2nd/200	3.07	286	1873	14
9534	IS-5 7069 + H5 (83)	3.21	1587	1679	81
9535	IS-6 7069 + H5 (80)	3.12	1600	1770	84
9536	IS-7 7003	5.80	175	1982	4
9537	1st R-1 + 1st Toluene	-	891	344	-

The ^{13}C values were 2.5 the normal value in the ratio (R-1) had and therefore this correction must be made. Average R-1 value is 2.58 $\therefore$.

$$\frac{2.5}{2.58} \times 1.504 \times 2000 = 291$$

Use factor $\times 322/334 \div wt = ppb$

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Nº 1832141

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2790ms

All other isomers were quantitated by Airglow. Notation of these isomers to be 2,3,7,8-TCDD. The notation as follows

7003	9*	ND	0.8	4	ND	4	2378	3:1
7069	50	4	14	22	6	14	2378	3:1
7001	15	1	5	5	2	5	1279	3:1
7001 up	-	-	-	-	7	13	1279	3:1
7032	ND	ND	ND	ND	ND	ND	1279	3:1
7069 (83.5)	47	4	17	26	8	86	1279	3:1
7069 (86.5)	26	4	14	20	8	840	1279	3:1
D.L. Find	0.7	0.7	0.7	0.7	0.7	0.7	1279	3:1
2	0.7	2	2	2	2	2	1279	3:1

* notation of these isomers was quantitated by Airglow. Notation of these isomers to be 2,3,7,8-TCDD. The notation as follows

could not be quantitated due to small levels of 2,3,7,8-TCDD present in sample.

For an estimate of average recovery the area for ¹³C = 1949 for 2 mly. ¹³C in the area the are area = 428 x 2.5 = 1070. For amt added (see pg 1832140). Missing for amt 2 to account for 4 mly injected 1070

$$\frac{1949}{2140} = 91\%$$

Note "C-TCDD again found was 493 ppb

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Ray W. Galt

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Analysis REPORT
TYPE OF REPORT

REPORT NO.: MDA-161

JOB/PROJECT NO.: 320.0100

DATE: 6 February 1981

PERIOD COVERED:

TITLE: GC/MS ANALYSIS OF THE TETRACHLORODIBENZO-P-DIOXIN
ISOMERS IN 2,4-DICHLOROPHENOLS

AUTHORS: F. D. Hileman
T. Mazer

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ABSTRACT: Four 2,4-dichlorophenol samples were analyzed in order to identify and quantitate any tetrachloro-dibenzo-p-dioxins present. Methodology, analytical results and quality assurance results are presented.

Technical approval: Frederick D. Hileman
Frederick D. Hileman
Sr. Research Chemist

Approved by: Robert F. Ivory
Robert F. Ivory
Environmental Analytical Sciences
Center Service Project Manager

Approval date: 2/6/81

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TITLE: GC/MS ANALYSIS OF THE TETRACHLORODIBENZO-P-DIOXIN
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Contributions of the following Dayton Laboratory personnel to this project are gratefully acknowledged:

Analyses: D. E. Kirk

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INTRODUCTION

Four 2,4-dichlorophenol samples were analyzed for the isomeric tetrachlorodibenzo-p-dioxins (TCDDs). The samples included two received from George Bautigam at MIC labeled KL01-7001:MB-833 and KK12-7069:MB-832 and two samples originally sent from the Krummrich Plant to us labeled KM01-7003 and KM07-7032. Sample 7003 had been found in previous work (R. F. Ivory, October 10, 1980 memo to J.A. Gloeckner) to contain at least two TCDDs at levels of 4.5 and 9.5 ppb while sample 7032 was found in the same study to be free of TCDD at a lower detection limit of 0.9 ppb and was used in this study as a blank. All samples were spiked with ^{13}C -labeled 2,3,7,8-TCDD to assist in both the qualitative and quantitative analysis of the TCDDs.

METHODOLOGY

Samples were prepared by dissolving in 20% KOH followed by extraction with petroleum ether. The ether extract was then cleaned up using concentrated base and concentrated sulfuric acid washes. The extract was then put through a final clean up using a Woelm basic alumina column. A tetradecane keeper was added to the eluate from the column and the solvent was blown down to a level of 200 μL and submitted for GC/MS analysis. This clean up procedure was necessary to reduce the impurities associated with the TCDD to a level such that capillary column analysis was possible while retaining adequate sensitivity.

The analysis was performed on a Hewlett Packard #5985B GC/MS system using a 50 meter Silar 10C capillary column for the TCDD separation as described by Buser et. al. Anal. Chem. 52, 1980 pages 2257 to 2263. Standards of the twenty two dioxin isomers were obtained from Professor Christopher Rappe, University of Umea, Sweden.

Quality assurance was carried out by having one replicate (Sample 7001) and by spiking Sample 7069 at the 83 ppb level and at the 860 ppb level with unlabeled 2,3,7,8-TCDD. These spiking levels were established from the suggestion given by Jim Wilson that the TCDD levels in these samples could approach 100 ppb. As mentioned previously, Sample 7032 served as the method blank.

RESULTS

The qualitative results of the analyses are graphically presented in the chromatographic traces of Figure 1. The top two traces are the standards of the 2:2 substituted TCDDs and the 3:1 and 4:0 substituted TCDDs. A typical selected ion chromatographic trace for Sample 7001 is given in Figure 2 showing the ion traces characteristic for unlabeled and ^{13}C -labeled TCDD. From Figures 1 and 2 it can be discerned that the peak eluting after the 1,3,6,8-TCDD is most likely not a dioxin due to the incorrect 320/322 ratio. Note that this peak does appear in Sample 7032, a sample which was found to be free of TCDDs. Observe also that the peak between the 1,3,7,8- and 1,3,6,9-TCDD is in all likelihood not a dioxin, again from the 320/322 ratio and note that again this peak appears in the 7032 sample. The presence of the 2,3,7,8-TCDD in the 7001 sample (Figure 2) and in Samples 7069 and 7003 is confirmed by the coelution with the ^{13}C -labeled 2,3,7,8-TCDD. Further evidence for the presence of 2,3,7,8-TCDD is shown in Figure 3 where Sample 7069 has been spiked with 83 and 860 ppb of the 2,3,7,8-TCDD and the corresponding peak has grown appreciably.

The cluster of TCDDs eluting after 2,3,7,8-TCDD consists of 3:1 substituted TCDDs. No attempt was made at this time to resolve this cluster, however, this can be done by using a different type of capillary column.

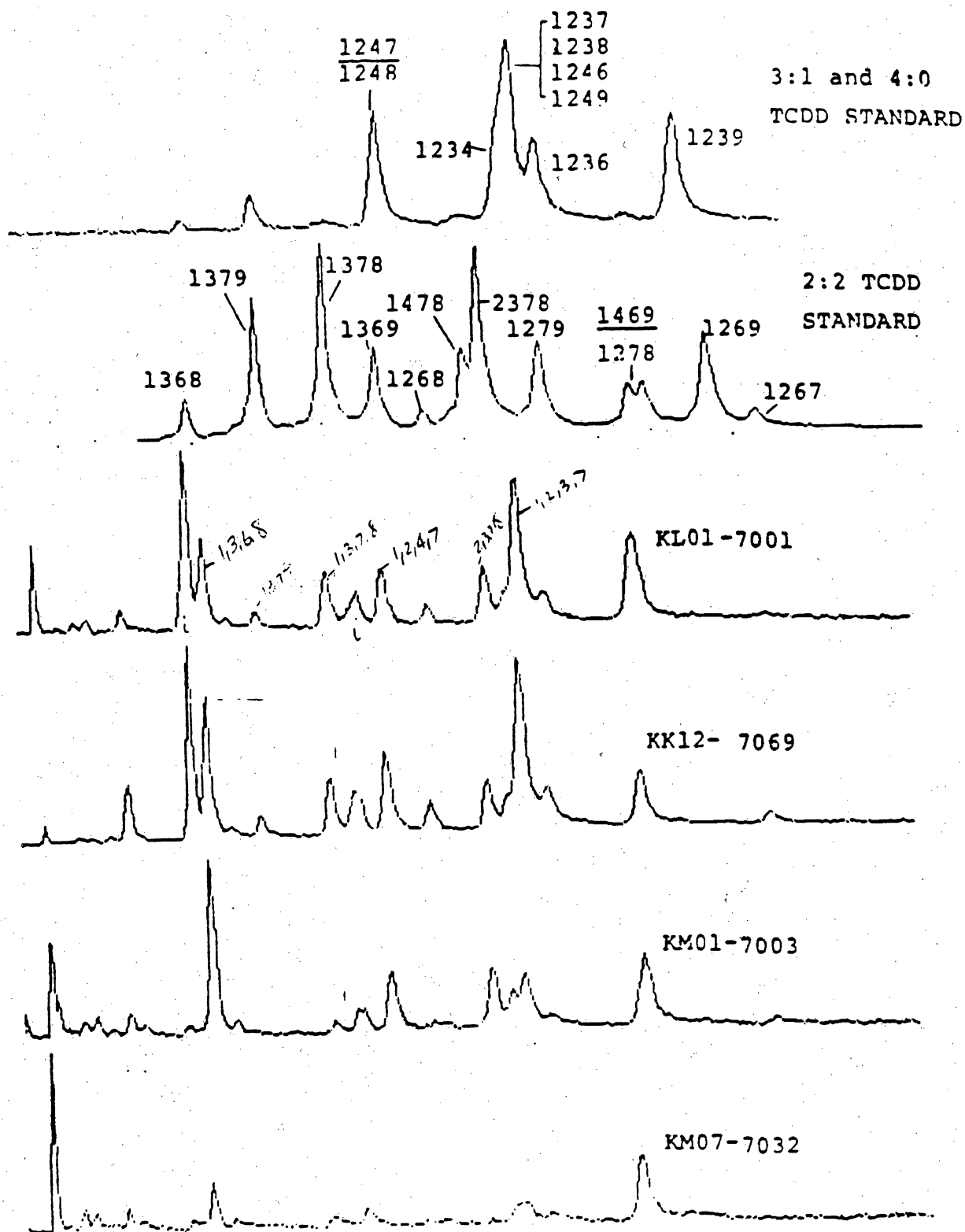


Figure 1. GC/MS analysis of TCDD isomers in 2,4-dichlorophenol.

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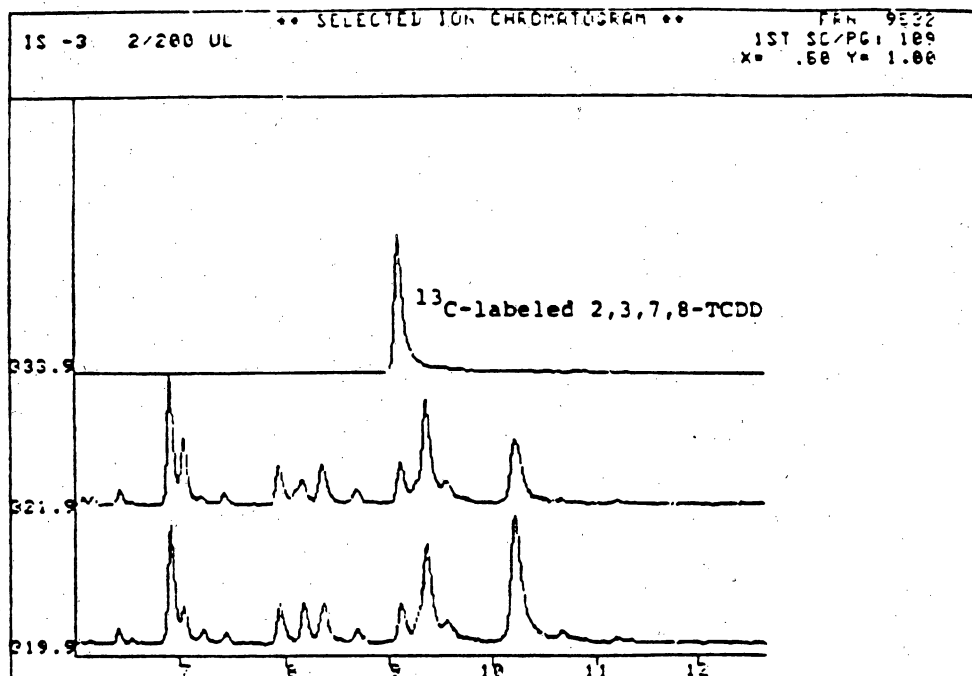


Figure 2. GC/MS analysis of the TCDD isomers in 2,4-dichlorophenol Sample KL01-7001.

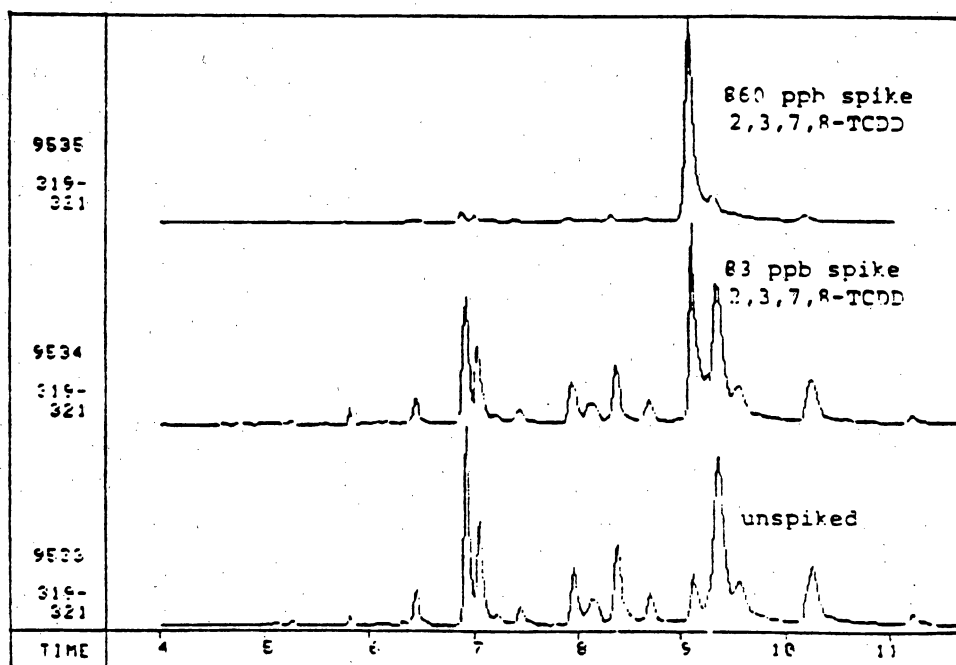


Figure 3. GC/MS analysis of unspiked and spiked 2,4-dichlorophenol Sample KK12-7032.

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The TCDD peak eluting with either the 1,2,7,8-or 1,4,6,9-TCDD is either not a dioxin or contains a significant amount of an interfering material. This is again evident from the 320/322 ratio (Figure 2) which is 1.94 rather than the actual value of 0.78.

The quantitative results for these analyses are given in Table 1. These results were obtained using the ^{13}C -labeled 2,3,7,8-TCDD as the internal standard to quantitate the 2,3,7,8-TCDD. All other TCDD isomers were then quantitated as if their response were identical to 2,3,7,8-TCDD.

The analysis of the replicate of 7001 was limited since a portion of the eluant from the Woelm column was spilled losing many of the TCDD isomers. The quantitation for the 2,3,7,8-TCDD is given since the presence of the internal standard would compensate for any losses in the 2,3,7,8-TCDD isomer. As a further indication of the reproducibility of the methods, the quantitative analyses of the other TCDD isomers in the 2,3,7,8-TCDD spiked samples are given in Table 1. Percent recoveries have not been calculated since an internal standard was used. The absolute recovery for the ^{13}C -labeled 2,3,7,8-TCDD spiked into the samples at 93 ppb was 91% $\pm$ 10%. The lower detection limits that are given were based on an evaluation of the method blank. Note that the detection limits have been raised for the 3:1 mixed isomers and the 1469/1278 isomers due to interferences in the method blank.

TABLE 1. QUANTITATIVE ANALYSIS OF TETRACHLORODIBENZO-P-DIOXIN ISOMERS IN 2,4-DICHLOROPHENOL

Sample	Specific TCDD isomer concentration, ng/g (ppb)							
	1368	1379	1378	1369	1268	2378	3:1 mix	1469 1278
KM01-7003	9 ^a	ND	0.8	4	ND	4	4	ND
KK12-7069	50	4	14	22	6	14	39	6
KL01-7001	15	1	5	5	2	5	13	2
KL01-7001	- ^b	-	-	-	-	7	-	-
KM07-7032	ND	ND	ND	ND	ND	ND	ND	ND
Detection limit	0.7	0.7	0.7	0.7	0.7	0.7	2	0.7
KK12-7069 (83 ppb 2,7,3,8-TCDD spike)	47	4	17	26	8	86	52	8
KK12-7069 (860 ppb 2,3,7,8-TCDD spike)	26	4	14	20	9	840	≠ ^c	≠

^aRetention time incorrect.

^bAnalysis of these isomers not carried out.

^cCould not be quantitated due to overlap from the high level 2,3,7,8-TCDD spike.