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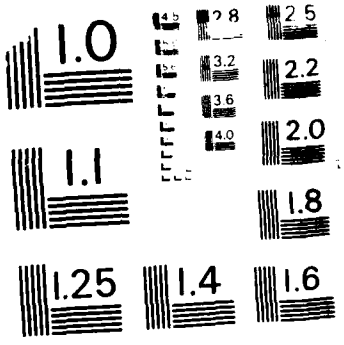

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Measurement of Pyridostigmine Bromide in Rodent Chow

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Measurement of Pyridostigmine Bromide in Rodent Chow (Toxicology Series 190)--Ferraris et al.

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An assay for the quantitation of pyridostigmine bromide (PYR) in ground rat chow is described. Pyridostigmine was extracted with water and measured by HPLC. The assay was linear from 0.01 to 2.0 mg pyridostigmine/g chow; the recovery of PYR from the chow was greater than 95%; interday variability was less than 3%; intraday variability was less than 2%. The distribution of PYR in chow was very homogeneous (CV < 3%) and PYR was stable in ground chow for at least 31 days at room temperature.

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ABSTRACT

An assay for the quantitation of pyridostigmine bromide (PYR) in ground rat chow is described. Pyridostigmine was extracted with water and measured by HPLC. The assay was linear from 0.01 to 2.0 mg pyridostigmine/g chow; the recovery of PYR from the chow was greater than 95%; interday variability was less than 3%; intraday variability was less than 2%. The distribution of PYR in chow was very homogeneous (CV ≤3%) and PYR was stable in ground chow for at least 31 days at room temperature.

Measurement of Pyridostigmine Bromide in Rodent Chow--Ferraris et al

In order to evaluate the 180-day oral subchronic toxicity of pyridostigmine in rats, it was necessary to administer the test compound in the diet. Satisfactory incorporation of pyridostigmine bromide (PYR) into ground rat chow requires that the test substance be mixed uniformly at the correct concentration and remain stable in the feed for the desired time period. To assess these parameters, it was first necessary to develop an assay for PYR in rat chow and then use the assay to determine the homogeneity and stability of PYR mixed in ground rat chow.

This report describes an assay for the quantitation of PYR in ground rat chow in which PYR was extracted from the chow with water and measured by High Performance Liquid Chromatography (HPLC) with UV detection. This assay was then used to assess the homogeneity and stability of PYR in rat chow which had been mixed in accordance with SOP OP-STX-106, "Diet Preparation for Feeding Studies." The desired concentration of PYR in the chow for the feeding study and thus for this report ranged from 0.01 mg PYR/g to 2 mg PYR/g chow.

MATERIALS AND METHODS

Equipment:

The chromatographic system used consisted of a Hewlett-Packard (Santa Clara, CA) 1090 liquid chromatograph with a Hewlett Packard 853 Personal Computer and DPU Multichannel Integrator with ThinkJet Printer. An Eberbach Mechanical Shaker (Ann Arbor, MI) and an IEC PR 6000 Centrifuge (Needham Heights, MA) were used in the extraction procedure.

Reagents:

Solvents were HPLC grade and chemicals were reagent grade. Acetonitrile was obtained from EM Science (Cherry Hill, NJ). The water used in preparation of all HPLC solutions was deionized, distilled, and purified of organics utilizing an Organicpure water purifier by Barnstead (Boston, MA). Tetramethylammonium chloride and 1-heptanesulfonic acid, sodium salt, were obtained from Aldrich Chemical Company (Milwaukee, WI); sodium phosphate monobasic was obtained from JT Baker Chemical Company (Phillipsburg, NJ). Pyridostigmine bromide, lot 525013, was supplied by Walter Reed Army Institute of Research (Washington, D.C.).

HPLC parameters:

The HPLC parameters that gave the optimum results are as follows:

Column:	Brownlee silica 5 um (100 x 4.6 mm)
Guard column:	Brownlee New Guard Silica 7 um
Flow:	1.5 ml/min
Buffer:	0.01 M Heptanesulfonic acid
	0.01 M Sodium dihydrogen phosphate
	0.0025 M Tetramethylammonium chloride
	Deionized, distilled water pH adjusted to 3 with sulfuric acid
Mobile Phase:	20% Acetonitrile, 80% Buffer
Wavelength: (Bandwidth)	269 (10) nm; ref wavelength, 350 (10) nm
Chartspeed:	2 cm/min
Run Time:	3.5 min
Peakwidth:	0.2 min
PYR Retention Time:	2.5 min

Preparation of Stock Solutions:

A stock solution of PYR was prepared by dissolving 50 mg of pyridostigmine bromide in 5 ml of water to give a final concentration of 10 mg PYR/ml (Stock Solution 1). This solution was diluted 10-fold to give a second stock solution with a concentration of 1 mg PYR/ml (Stock Solution 2). Each solution was divided into 500-ul portions, placed in plastic microcentrifuge tubes, and stored in the freezer (-4°C) for subsequent use. These solutions were used to spike the blank rat chow and water samples.

Extraction of PYR from Rat Chow:

The following procedure was used for extracting PYR from rat chow samples containing concentrations of 0.1-2 mg PYR/g chow:

One gram of chow was weighed into a 50-ml plastic centrifuge tube. The PYR was extracted from the chow by adding 35 ml of water, shaking in a mechanical shaker for 15 minutes, centrifuging and pouring the supernatant into a 200-ml volumetric flask; this procedure was performed four times. The combined supernatants were brought to volume with water and mixed well. A small portion of the combined supernatants (1-2 ml) was filtered (0.2-um membrane filter) prior to HPLC analysis.

The following procedure was used for rat chow samples containing lower concentrations of PYR (0.01-0.1 mg/g chow):

One gram of 0.1 mg PYR/g chow or 2 g of 0.01 mg PYR/g chow was weighed into a plastic centrifuge tube. The PYR was extracted from the chow twice by adding 25-ml aliquots of water, shaking in the mechanical shaker for 30-40 minutes, and centrifuging. The supernatants were combined in a 50-ml volumetric flask, brought to volume with water, and mixed well. A small portion of the combined supernatant (1-2 ml) was filtered twice, first through a 0.45-micron filter and then through a 0.2-micron filter, prior to analysis.

Preparation of Spiked Rat Chow for Standard Curve:

The five concentrations of PYR in rat chow used for the standard curve were prepared by adding various amounts of PYR stock solution to rat chow as shown in Table 1:

Table 1

Preparation of Standard Curve

Level No.	Concentration (mg PYR/g chow)	Chow (g)	Amount of Stock Solution (ul)	Stock Solution No.
1	2.00	1	200	1
2	1.00	1	100	1
3	0.50	1	50	1
4	0.10	1	100	2
5	0.01	2	20	2

The spiked samples were analyzed either on the day of preparation or the next. The standard curve was determined by performing linear regression analysis of the peak height versus nanograms of PYR injected on the column. The concentration of PYR in each sample was then calculated from the standard curve and the dilution factor. All statistical calculations were performed on a Data General mv 8000 minicomputer using Minitab Software(1).

Homogeneity & Stability:

All rat chow containing PYR was prepared with the aid of a Twin Shell Blender (Patterson-Kelly, East Stroudsburg, PA) in accordance with SOP OP-STX-16 "Diet Preparation for Feeding Studies." Homogeneity samples were taken from the left, right, and bottom ports of the Twin Shell Blender and analyzed in duplicate or triplicate. The sample size for the homogeneity studies was 1 g for levels 1-4 and 2 g for level 5. Chow samples for stability studies were analyzed on the day of preparation (Day 1) and stored in glass beakers covered with parafilm at room temperature for the duration of the study.

RESULTS AND DISCUSSION

Under chromatographic conditions described above, pyridostigmine elutes at a retention time of 2.6 minutes. The HPLC traces obtained from the extraction of blank rat chow are shown in Figures 1 and 2; no peaks that might interfere with PYR are present. The chromatograms obtained from the extraction of PYR from rat chow at various concentrations are shown in Figures 3-6.

Points on the standard curve were determined by analyzing samples of blank chow spiked with PYR at five concentrations: 2.0, 1.0, 0.5, 0.1, and 0.01 mg PYR/g of chow. When the spiked chow is extracted according to the above procedure, these concentration levels correspond to the following nanograms of PYR injected on column: 250, 125, 62.5, 50, and 10. Values from a typical standard curve (Table 2) show that the assay is linear ($r=0.9999$) over the range of 10-250 ng PYR on column.

Table 2

Standard Curve

Concentration (mg PYR/g chow)	PYR on Column (ng)	Peak Height (mAU)	SD	CV(%)
2.00	250	18.490	± 0.473	2.5
1.00	125	9.239	0.153	1.6
0.50	62.5	4.659	0.10	2.3
0.10	50	3.601	0.104	2.9
0.01	10	0.700	0.027	3.8

Slope = 0.074155; Intercept = 0.000599; Correlation coefficient (r) = 0.9999

Since the spiked samples for the standard curve were not always analyzed on the day of preparation, it was necessary to determine the overnight stability of the spiked samples. A comparison of the values obtained on day 1 and day 2 (Table 3) show that PYR is stable in the chow for at least 24 hrs.

Table 3

PYR Stability - Overnight

Concentration (mg PYR/g chow)		
	Observed	
Targeted	Day 1	Day 2
2.00	2.036	2.072
1.00	0.988	1.008
0.50	0.522	0.502
0.10	0.1012	0.0938
0.01	0.0110	0.0106

Extraction recoveries were determined by comparing peak heights from the chromatograms obtained from the extraction of spiked rat chow with the aqueous standards analyzed on the same column. Table 4 shows that recoveries are well above 90% and comparable.

Table 4

Extraction Recoveries

Concentration (mg PYR/g chow)	% Recovery	SD	N
2.00	97.67	± 1.6	6
1.00	97.83	1.5	6
0.50	100.00	1.6	6
0.10	97.00	2.0	10
0.01	96.25	2.8	12

Assay variability studies were conducted over a five-day period at all five concentrations; the interday variability (Table 5) was less than 3%. The intraday variability at the two lowest concentrations was less than 2%.

Table 5

Assay Variability

Concentration (mg PYR/g chow)			
Targeted	Observed	SD	CV (%)
Interday (n=5)			
2.00	2.004	±0.056	2.6
1.00	0.992	0.0097	1.3
0.50	0.509	0.0146	2.9
0.10	0.0974	0.0025	2.6
0.01	0.0107	0.0002	1.8
Intraday			
0.10[a]	0.0934	0.0010	1.1
0.01[b]	0.0114	0.0002	1.7

[a] n=4; [b] n=6

[illegible]geneity studies were performed at all concentrations. [illegible]y the results in Table 6, the chow mixed in the blend[illegible] [illegible]neous at all concentration levels. At any concentration, [illegible]efficient of variation for feed samples taken from ten [illegible]ocations in the blender is less than 3%.

Table 6

Homogeneity

Concentration (mg PYR/g chow)	Observed (mean value)	SD	CV(%)	Range (mg PYR/g chow)	
[illegible]	[illegible].977	±0.0170	.8	1.961 - 2.005	6
[illegible]	0.973	0.0125	1.3	0.958 - 0.986	6
[illegible]	0.504	0.0078	1.5	0.516 - 0.493	6
0.1[illegible]	0.0966	0.0020	2.0	0.937 - 0.993	6
0.01	0.0[illegible]92	0.0003	2.9	0.00985 - 0.0178	6

[illegible]ings on the stability of PYR in rat chow at two [illegible] [illegible] a 31-day period at room temperature (Tab[illegible] [illegible] [illegible]ntration of PYR remains constant wi[illegible] [illegible] [illegible]its of the assay and of the diet preparat[illegible] [illegible] [illegible] study of Level 5 was performed be[illegible] [illegible] [illegible] study seemed to indicate a drop [illegible] [illegible] [illegible] from Day 1 to Day 7. The decrease in [illegible]ncentration [illegible] [illegible] [illegible] that the drop observed in the [illegible] [illegible] [illegible] of data in chronically mixed [illegible] [illegible] [illegible].

Table 7
Stability of Pyridostigmine
in Rat Chow (n=6)

Level		Observed Concentration (mg PYR/g chow)	SD
Level 5 (0.01 mg PYR/g chow)			
Trial 1	Day 1	0.0109	±0.0008
	Day 2	0.0104	0.0002
	Day 3	0.0102	0.0003
	Day 4	0.0102	0.0005
	Day 8	0.0103	0.0002
	Day 15	0.0102	0.0001
	Day 22	0.0102	0.0002
	Day 29	0.0109	0.0004
Trial 2	Day 1	0.0098	±0.0003
	Day 2	0.0103	0.0006
	Day 9	0.0098	0.0007
	Day 16	0.0096	0.0009
	Day 23	0.0100	0.0003
	Day 30	0.0098	0.0005
Level 4 (0.1 mg PYR/g chow)			
	Day 1	0.0966	±0.0021
	Day 2	0.0949	0.0016
	Day 9	0.0937	0.0010
	Day 15	0.0939	0.0016
	Day 24	0.0929	0.0015
	Day 31	0.0937	0.001

SUMMARY

The oral subchronic toxicity study of pyridostigmine in the rat requires that pyridostigmine be blended into ground rat chow. This report describes an assay in which pyridostigmine is extracted from rat chow with water and quantitated by HPLC analysis. The assay was demonstrated to be linear over the range of concentrations of PYR in rat chow (0.01-2.0 mg PYR/g chow) to be used in the toxicity study; the extraction recovery of PYR was greater than 95% at all concentration levels. The intraday assay variability was less than 2%, the interday variability was less than 3%. Using this assay, the homogeneity of PYR feed samples mixed with a Twin Shell Blender (SOP OP-STX-16) was assessed; the

results showed that the concentration of PYR mixed in the chow was homogeneous (CV < 3%) in all samples taken from different locations in the blender. The stability of PYR mixed in rat chow at two concentrations was also assessed over a 31-day period. The results showed that the concentration of PYR in the feed remained essentially unchanged after 31 days at room temperature.

References

1. Ryan TA Jr, BL Joiner, Ryan BF. Minitab Students Handbook. Boston: Duxbury Press, 1981.

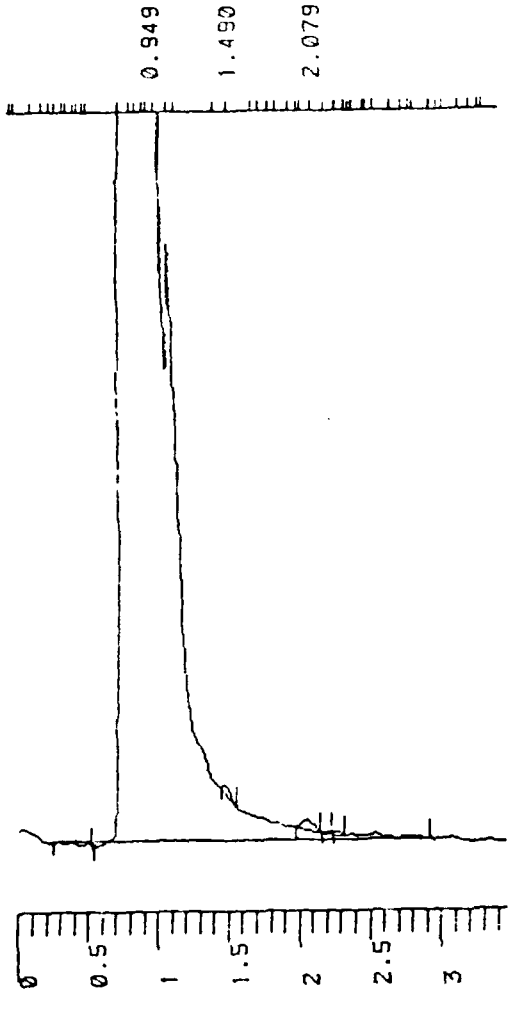

Fig. 1. Chromatogram of extracted blank rat chow (1 g chow/200 ml water)

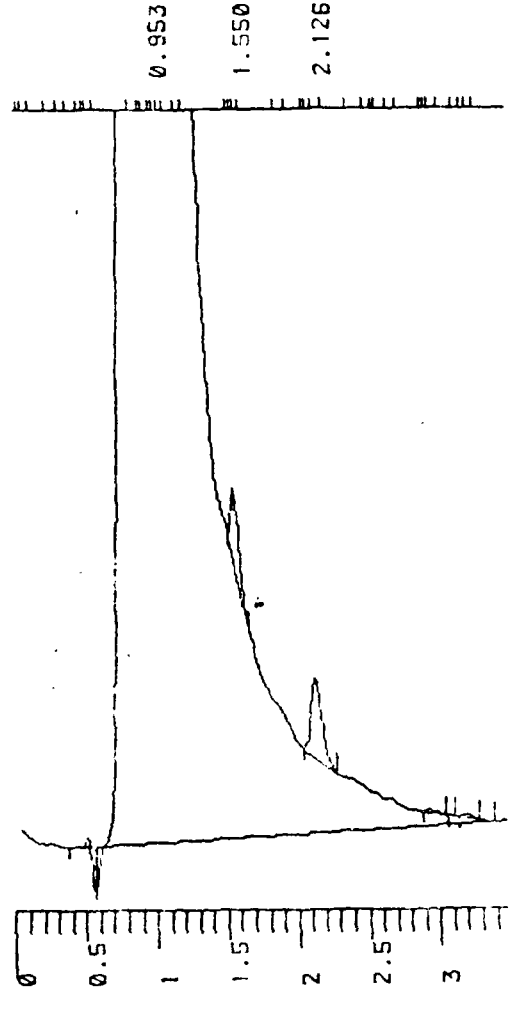

Fig. 2. Chromatogram of extracted blank rat chow (2 g chow/50 ml water)

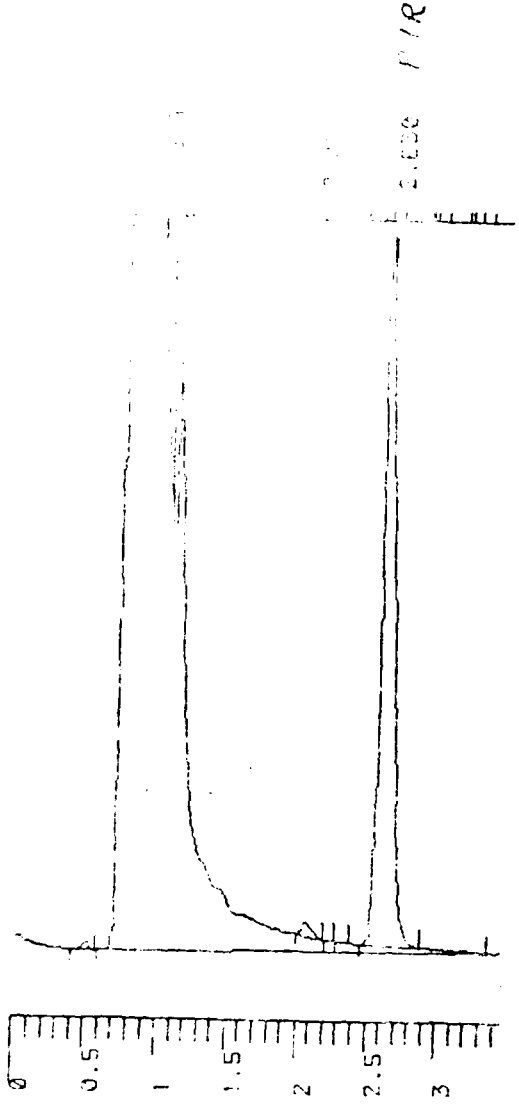

Fig. 3 Chromatogram of extracted rat chow (1 g chow/200 ml water), PYR concentration (1 mg/g chow), 125 ng PYR on column.

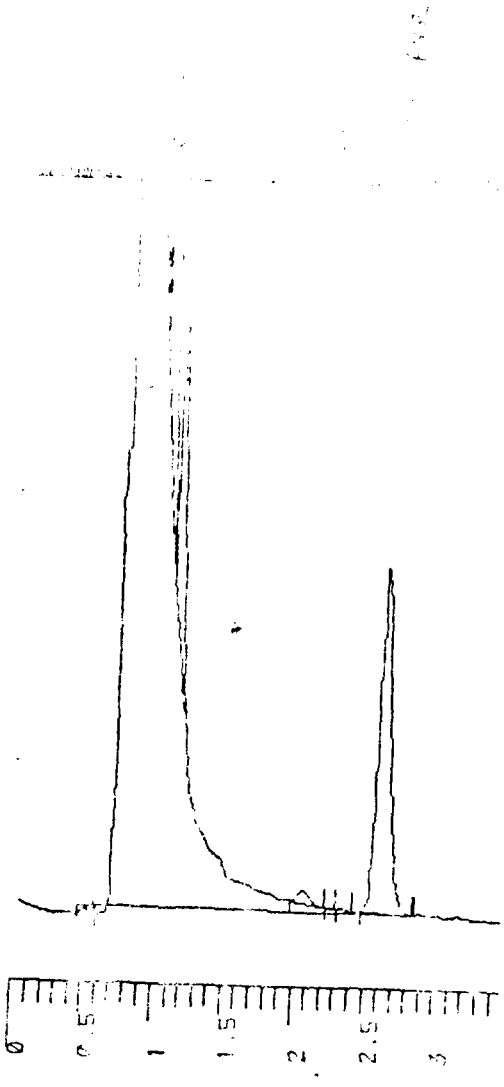

Fig 4. Chromatogram of extracted rat chow (1 g chow/200 ml water), PYR concentration (0.5 mg/g chow), 62.5 ng PYR on column

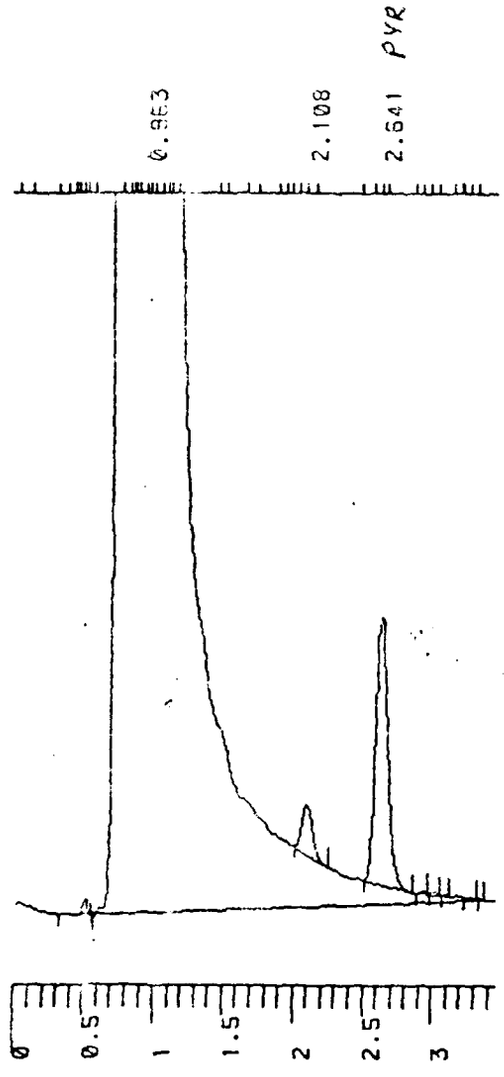

Fig. 5. Chromatogram of extracted rat chow (1 g chow/50 ml water), PYR concentration (0.1 mg/g chow), 50 ng PYR on column.

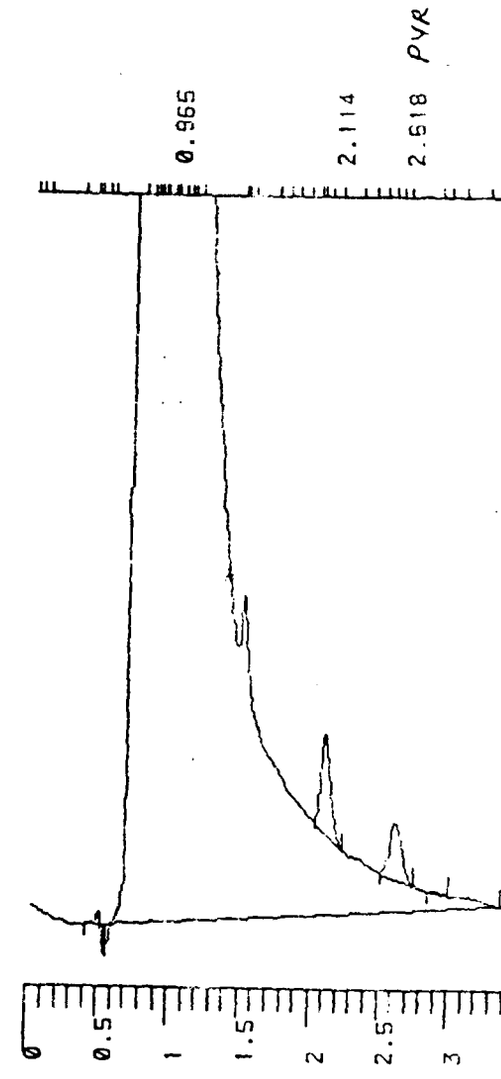

Fig. 6. Chromatogram of extracted rat chow (2g chow/ 50 ml water), PYR concentration (0.01mg/g chow), 10 ng PYR on column.

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