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Study Title

DETERMINATION OF DPX-Y6202, DPX-Y6202 ACID, AND ME-DPX-Y6202 RESIDUES IN BOVINE MILK

Data Requirement

U.S. EPA Pesticide Assessment Guidelines
Subdivision O, 171-4

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Performing Laboratory

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Laboratory Project ID

AMR-515-86

13A

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d) (1)(A), (B), or (C).

Company E. I. du Pont de Nemours and Company, Inc.

Company Agent Tony E. Catka (Typed Name) Date August 14, 1987

Registration Specialist (Title)

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GOOD LABORATORY PRACTICE STATEMENT

The GLP requirements specified in 40 CFR Part 160 are not applicable to residue data chemistry requirements at the time of submission.

This study was conducted in the spirit of good laboratory practices.

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TABLE OF CONTENTS

ABSTRACT

A procedure has been developed for the analysis of DPX-Y6202, DPX-Y6202 Acid, and ME-DPX-Y6202 in bovine milk. The milk was extracted with acetonitrile and the acetonitrile then evaporated. The oil residue was then hydrolyzed with a mixture of lipase and esterase enzymes to disassociate the fat and also convert DPX-Y6202 and ME-DPX-Y6202 to DPX-Y6202 Acid. The DPX-Y6202 Acid was then extracted from the aqueous enzyme solution with chloroform which was evaporated. The level of DPX-Y6202 Acid was determined by multi-dimensional HPLC with spectrophotometric detection at 335 nm.

INTRODUCTION

Ethyl-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy] propanoate, (DPX-Y6202), is the active ingredient in Assure® Herbicide. DPX-Y6202, with the common name, quizalofop ethyl, is effective in controlling grasses in broadleaf crops such as soybeans. Because soybeans may be used in dairy cattle feed we have developed a procedure to determine residues of DPX-Y6202 and metabolites in bovine milk. DPX-Y6202 has been shown by Steve Hundley (1)to be metabolized by goats to DPX-Y6202 Acid and to the methyl ester of DPX-Y6202 (ME-DPX-Y6202). The structures of DPX-Y6202 (ethyl-2-[4-

(6-chloroquinoxalin-2-yloxy)phenoxy] propanoate), DPX-Y6202 Acid ([4-(6-chloroquinoxalin-2-yloxy)phenoxy] propanoic acid), and ME-DPX-Y6202 (methyl-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy] propanoate) are given in Figure 1.

We have developed a procedure to determine these three compounds in milk. Both DPX-Y6202 and ME-DPX-Y6202 are converted to DPX-Y6202 Acid during the procedure and the DPX-Y6202 Acid measured by multi-dimensional HPLC. The procedure developed has been validated using a sample of milk from a goat treated with 14C-DPX-Y6202.

PROCEDURE

Equipment and Reagents

A Du Pont Model 8800 HPLC (E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware) consisting of microprocessor controller, Model 870 pump, a column oven, and a data system was used. The column oven was fitted with a Model 7000 Rheodyne switching valve (Rheodyne, Inc., Cotati, California). The pneumatic actuated switching valve was controlled from the data system through a Rainin Solenoid Interface (Rainin Instruments, Inc., Woburn, Massachusetts). Samples were injected with a Hitachi Model 655A-40 autosampler (EM Science, Gibbstown, New Jersey). The detector was a

Waters Model 481 (Waters Associates, Milford, Massachusetts) spectrometer set at 335 nm. The HPLC columns were a Sepralyte© C2, 4.6 mm x 15 cm, column (Analytichem International, Harbor City, California) and a Zorbax® Phenyl, 4.6 mm x 15 cm, column (E. I. du Pont de Nemours and Company).

For homogenization and extraction of samples, a Tekmar Tissumizer© (Tekmar Company, Cincinnati, Ohio), Model SDT-1810, with a Model SDT-182 EN shaft and generator was employed. A Vortex-Genie© mixer (Fisher Scientific Company, Pittsburgh, Pennsylvania) was used for mixing of samples in centrifuge tubes. An International Equipment Company Model K centrifuge (Fisher Scientific), fitted with a head to hold six 250 mL centrifuge jars, was used to centrifuge samples. A Precision Model 50 shaking water bath (GCA Corporation, Precision Scientific Group, Chicago, Illinois) was used to shake the samples at 37°C for the enzyme hydrolysis step.

For concentration of samples, a vacuum rotary evaporator with a water bath set at 50°C was used. Either 250 mL glass-stoppered enlenmeyer flasks or pear-shaped flasks with 24/40 ground-glass joints (Kontes, Vineland, New Jersey), #K-608700, were used on the rotary evaporator. An N-EVAP® evaporator (Organomation Associates, Worcester, Massachusetts) was used to concentrate samples in centrifuge tubes to dryness with nitrogen.

A Millipore all-glass filter apparatus, #XX15 047 00, with a 0.45 mm Durapore© filter, #HVLP 047 00, was used to filter the HPLC solvents (Millipore Corporation, Bedford, Massachusetts). Millipore Millex©-HV disposable Durapore© filters, #SJHV LO4 NS, were used to filter samples before they were injected on the HPLC.

The lipase enzyme from Rhizopus Arrhizus, #437706, was purchased from Behring Diagnostics, La Jolla, California in bottles containing 100,000 units. The esterase enzyme from porcine liver, #E3128, was purchased from Sigma Chemical Co., St. Louis, Missouri in bottles containing a 30 mg suspension in 3.2 M ammonium sulfate, pH 8. The activity of the enzyme was 260 units/mg and was dissolved in 2.8 mL of the ammonium sulfate solution. Both enzymes were stored at 0 to 4°C until they were used.

The standards of DPX-Y6202 (purity = 99.7%), DPX-Y6202 Acid (purity = 97.7%), and ME-DPX-Y6202 (purity = 96.6%) were obtained from the Agricultural Products Department, E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware. All solvents were distilled-in-glass HPLC grade obtained from Fisher Scientific. The chloroform was stablized with 0.75% ethanol. All other chemicals were A.C.S. reagent grade obtained from either Fisher Scientific or VWR Scientific, San Francisco, California.

Buffer X was made by dissolving 12.6 grams of trizma hydrochloride (Sigma Chemical Co., St. Louis, Missouri), 0.2 grams

of cholic acid (Sigma Chemical Co.), and 11.8 grams of calcium chloride dihydrate in 2.0 L distilled water. The pH was then adjusted by monitoring with a pH meter to 7.8 with 1 N sodium hydroxide. Buffer Y was made by dissolving 105.7 grams of ammonium sulfate and 0.44 gram of dibasic potassium phosphate in 250 mL distilled water. The pH of this buffer was adjusted by monitoring with a pH meter to 6.0 with concentrated phosphoric acid.

A lipase enzyme solution was then made by adding 2.7 mL of buffer Y to a 100,000 unit bottle of lipase. The bottle was rolled gently to suspend the enzyme and was stable for two weeks after it was suspended in the buffer as long as it was stored at 0 to 4°C.

The two HPLC mobile phases (see Table 1) were made by mixing together the proportions in Table 2 of acetonitrile and pH 2.2 phosphoric acid solution. The pH 2.2 phosphoric acid solution was made by adjusting the pH of distilled water to 2.2 with concentrated phosphoric acid. The pH was monitored with a pH meter. The prepared mobile phases were then filtered with a 0.45 mm Durapore® filter before using. A pH 8.5 solution was made by adjusting the pH of 500 mL distilled water to 8.5 ± 0.5 using 1 N NaOH. Other solutions were made in the proportions given in Table 3.

Isolation and Clean Up

Each milk sample was thawed in the shaker bath at 37°C immediately before it was needed. It was then shaken well, a 10

gram aliquot weighed into a 250 mL glass centrifuge bottle and the rest of the sample returned to the freezer. 150 mL acetonitrile was added, the sample homogenized for 1 min. with the Tissumizer© homogenizer, and the shaft and generator rinsed well with acetonitrile which was collected in the centrifuge bottle. After centrifuging for 10 min., the liquid was decanted into a 250 mL glass-stoppered erlenmeyer flask, and the volume reduced to about 30 mL on the rotary evaporator.

The milk solids in the centrifuge bottle were re-extracted with 120 mL acetonitrile as described above. The acetonitrile extract was added to the erlenmeyer flask with the remaining acetonitrile from the first extraction and the sample then concentrated to dryness on the rotary evaporator. 100 mL of buffer X was added to each sample in the erlenmeyer flask and it was then shaken and ultrasonically mixed to emulsify the fat. After the fat was emulsified, 7500 units of the lipase enzyme solution (200 mL) and 300 units of the esterase solution (115 mL) was added. The samples were then incubated on the shaker bath for at least 12 hours at 37°C. The shaking action was adjusted to 100-120 shakes/minute.

After the samples had incubated for at least 12 hours, the contents of the erlenmeyer flask was transferred to a 500 mL separatory funnel and 5 mL of 10% hydrochloric acid added. Each erlenmeyer flask was then rinsed twice with 25 mL of acetonitrile each time. Each rinse was ultrasonically mixed and shaken in the

flask and then also transferred to the separatory funnel. An additional 150 mL rinse of the erlenmeyer flask with chlorform was made and this also transferred to the separatory funnel. After the contents were shaken vigorously for 1 minute, and the two layers had separated, the bottom layer was drained into a 500 mL pear-shaped flask and then concentrated to dryness on a rotary evaporator at 45°C. The top layer was discarded.

Each sample was then transferred from the pear-shaped flask to a 500 mL separatory funnel with 3 x 35 mL rinses of acetonitrile followed by 3 x 45 mL rinses of hexane. The rinses were ultrasonically mixed and shaken as needed to remove the residue off the walls of the pear-shaped flask. Each separatory funnel was then shaken vigorously for 1 minute, and the bottom acetonitrile layer drained into a 500 mL pear-shaped flask. More acetonitrile (100 mL) and hexane (25 mL) was added to each separatory funnel, the funnel shaken for 1 minute, and the bottom acetonitrile layer drained into the pear-shaped flask with the acetonitrile from the first extraction.

Each sample was then concentrated to dryness on a rotary evaporator at 45°C. The residue for each sample was then transferred to a 13 mL glass-stoppered centrifuge tube with 3 x 3 mL rinses of the pear-shaped flask with solution A (see Table 3). Again, the rinses were ultrasonically mixed and shaken as needed to remove the residue off the walls of the pear-shaped flask. The

solvent was then evaporated from each sample in the centrifuge tube using the N-EVAP® nitrogen evaporator with the water bath at room temperature. Each sample was then stored at 0 to 4°C until it was prepared for analysis on the HPLC.

Liquid Chromatography

Samples were quantitated by multidimensional HPLC using a combination of two columns. A diagram of the columns and switching valve arrangement is shown in Figure 2 where the first column (C_1) was the Sepralyte® C2 column and the second column (C_2) was the Zorbax® Phenyl column. In valve position I, the effluent from C_1 went through a 10 mL bypass loop, back to the valve, and to the detector. In the other position, II, the effluent from C_1 went to the valve, to C_2, back to the valve, and then to the detector.

Table 1 gives a typical timing sequence for analysis of samples. At the time of injection, the valve was in position I and C_2 was bypassed. When DPX-Y6202 Acid started to elute from C_1, the valve was switched to position II to trap the peak on C_2. After the peak was trapped, the valve was switched back to position I. The valve switching times were set at -0.35 and +0.10 min. around the retention time for DPX-Y6202 Acid on C_1 which was determined each morning.

After DPX-Y6202 Acid had been trapped on C_2, the mobile phase was changed from solution I to solution II and the flow rate increased from 1.0 mL/min. to 2.5 mL/min. to quickly equilibrate C_1 to the new mobile phase and to clean the rest of the sample off. After C_1 had equilibrated to the stronger mobile phase, the flow rate was decreased to 1.0 mL/min and the valve switched to position II to elute DPX-Y6202 Acid from C_2. After all peaks had eluted from C_2, the valve was switched to position I, the mobile phase changed to solution I, and the flow rate increased to 2.5 mL/min. to quickly equilibrate C_1 to solution I. After C_1 had equilibrated, the flow rate was decreased to 1.0 mL/min. and the next sample or standard injected.

The detector attentuation was set at 0.002 AUFS and the time constant at 5 sec. The data system attentuation was set at 1, the oven temperature at 50°C, and the autosampler injection volume at 50 mL.

For HPLC analysis, each sample was redissolved in 1.0 mL of acetonitrile, ultrasonically mixed, and vortex mixed. Then 3.0 mL hexane was added, the sample vortex mixed for 30 seconds, and centrifuged for 5 minutes to separate the layers. The top hexane layer was removed with a pastuer pipette and discarded. The wash with 3.0 mL hexane was repeated again and the hexane discarded. Then 1.0 mL of pH 8.5 solution was added and the sample vortex mixed for 30 seconds. The bottom aqueous layer was then removed with a

pastuer pipette, transferred to a 2.0 mL disposable syringe, and filtered through the Millex® disposable filter into an autosampler vial. Samples were then injected on the HPLC intermixed with standards.

Standards

Stock standards. Stock standards for DPX-Y6202, DPX-Y6202 Acid, and ME-DPX-Y6202 were each made at 100 mg/mL by dissolving 10 mg of each in 100 mL of solution H (see Table 3).

Fortifying standards. Intermediate stock standards of each were made at 10.0 mg/mL by diluting 5.0 mL of the stock standards to 50 mL with solution H. Fortifying standards at 1.0 mg/mL and 0.2 mg/mL were made by diluting 10.0 or 2.0 mL of the 10.0 mg/mL standards to 100 mL with solution H.

HPLC standards. A 10.0 mg/mL standard was made by placing 5.0 mL of the stock DPX-Y6202 Acid standard in a 50 mL volumetric flask, evaporating the solvent, and diluting to volume with solution II (see Table 2). Standards at 1.0, 0.5, 0.2, and 0.1 mg/mL were made by pipetting 10.0, 5.0, 2.0, and 1.0 mL of the 10 mg/mL standard into 100 mL volumetric flasks and making to volume with solution II. A 0.05 mg/mL standard was made by pipetting 5.0 mL of the 1.0 mg/mL standard into a 100 mL volumetric flask and making to

volume with solution II. The 0.05, 0.1, and 0.2 standards were then injected on the HPLC intermixed with the samples.

Calculations

The sensitivity for each standard injected on the HPLC, S, in (mm-mL)/mg units, was calculated by the equation;

$$S = \frac{P_S}{C_S} \qquad (1)$$

where, P_S was the peak height of the standard in millimeters and C_S was the concentration in mg/mL units. The average sensitivity for standards injected interspersed with samples, S_a, was calculated and used for calculation of sample concentrations.

The sample concentration, C, of DPX-Y6202 Acid, in mg/g units (ppm), was calculated using the equation;

$$C = \frac{(P)(V)}{(W)(S_a)} \qquad (2)$$

where, P was the sample peak height in millimeters, V was the final sample volume in mL (2.0), and W was the sample weight in grams (10.0). For samples fortified with DPX-Y6202 or ME-DPX-Y6202, the

concentration of DPX-Y6202 Acid was converted to DPX-Y6202 or ME-DPX-Y6202 by multiplying by the molecular weight ratios of 1.08 and 1.04 respectively.

RESULTS AND DISCUSSION

Recovery data for milk and skim milk samples fortified with DPX-Y6202, DPX-Y6202 Acid, and ME-DPX-Y6202 are given in Tables 4 and 5. Recoveries averaged 87% (s=9%) for 10 milk samples and 93% (s=6%) for 3 skim milk samples fortified with DPX-Y6202. They averaged 78% (s=8%) for 10 milk samples and 88% (3%) for 3 skim milk samples fortified with DPX-Y6202 Acid, and 81% (s=7%) for 6 milk samples and 81% for 2 skim milk samples fortified with ME-DPX-Y6202.

Chromatograms of a control pm milk sample, the same sample fortified at 0.01 ppm with DPX-Y6202, and a 21 day pm milk sample from a cow treated with 5.0 ppm DPX-Y6202 are shown in Figures 3, 4, and 5. Chromatograms of a control skim milk sample, the same sample fortified at 0.02 ppm with DPX-Y6202 Acid, and a 16 day skim milk sample from a cow treated with 5.0 ppm DPX-Y6202 are shown in Figures 6, 7, and 8.

The procedure was validated by processing two fifth day milk samples from a goat treated with [phenyl-^{14}C] DPX-Y6202 (P-label) and two fifth day samples from a goat treated with [quinoxaline-phenyl-^{14}C] DPX-Y6202 (QP-label) (1). Radioactivity levels were measured by scintillation counting throughout the analysis of the samples to determine where any recovery losses occurred. Unextracted residues were found to be 2% of the total radioactivity for the P-label milk and 8% for the QP-label milk. Of the extracted radioactivity, expressed as DPX-Y6202 equivalents, 80% was measured by HPLC as DPX-Y6202 Acid for the P-label milk and 85% for the QP-label. This loss of 20% for the P-label milk includes 2% lost into the aqueous phase of the chloroform partition and 12% into the hexane wash of the HPLC sample extract. The 15% loss for the QP-label milk includes 10% lost into the aqueous phase and 7% into the hexane wash. Therefore, this procedure determines greater than 80% of DPX-Y6202, DPX-Y6202 Acid, and ME-DPX-Y6202 extracted from goat milk as DPX-Y6202 Acid.

A significant part of the radioactivity extracted from milk, about 50%, is associated with the fat in the milk and is lost into the hexane wash of acetonitrile if lipase and esterase enzymes are not used. The combination of enzymes is used to hydrolyze the fat and thereby release the radioactivity. When the enzymes are used, the amount of radioactivity associated with the fat and lost in a hexane wash is reduced to less than 15%.

The amount of cholic acid added to the enzyme hydrolysis step as an emulsifier is critical since too much inhibits the hydrolysis reaction. We have found that 0.01% as used in the procedure and 0.02% both do not significantly inhibit the reaction but that 0.05% does. We ran the enzyme reaction overnight since that was convenient, but the reaction is probably completed in a shorter time.

REFERENCES

1. Hundley, S. G., "Metabolism of ^{14}C-Labeled DPX-Y6202 by Lactating Goats", E. I. Du Pont De Nemours and Company, Inc., Wilmington, Delaware, Agricultural Products Department, Document #AMR-618-86.

TABLE 1

TYPICAL HPLC TIMING SEQUENCE

Time Range (Min)	Mobile Phase	Flow Rate (mL/Min)	Valve Position	Columns Used
0.00 to 6.80	Solution I	1.0	I	C_1
6.80 to 7.25	Solution I	1.0	II	$C_1 + C_2$
7.25 to 8.00	Solution I	1.0	I	C_1
8.00 to 18.00	Solution II	2.5	I	C_1
18.00 to 19.50	Solution II	1.0	I	C_1
19.50 to 30.00	Solution II	1.0	II	$C_1 + C_2$
30.00 to 43.00	Solution I	2.5	I	C_1

TABLE 2

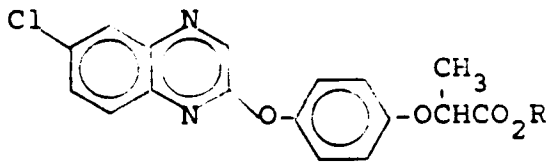
AQUEOUS SOLUTION COMPOSITIONS

Solution	Percent Acetonitrile	Percent pH 2.2 Phosphoric Acid
I	40	60
II	50	50

TABLE 5

SKIM MILK RECOVERIES

Compound	Number of Samples	Spike Range (ppm)	Recovery Range (%)	Average Recovery (%)	Standard Deviation (%)
DPX-Y6202	3	0.01 - 0.05	87 - 98	93	6
DPX-Y6202 Acid	3	0.01 - 0.05	85 - 90	88	3
ME-DPX-Y6202	2	0.01 - 0.02	70 - 92	81	--



R = C_2H_5 for DPX-Y6202
R = H for DPX-Y6202 Acid
R = CH_3 for ME-DPX-Y6202

FIGURE 1. Structures of DPX-Y6202, DPX-Y6202 Acid, and ME-DPX-Y6202.

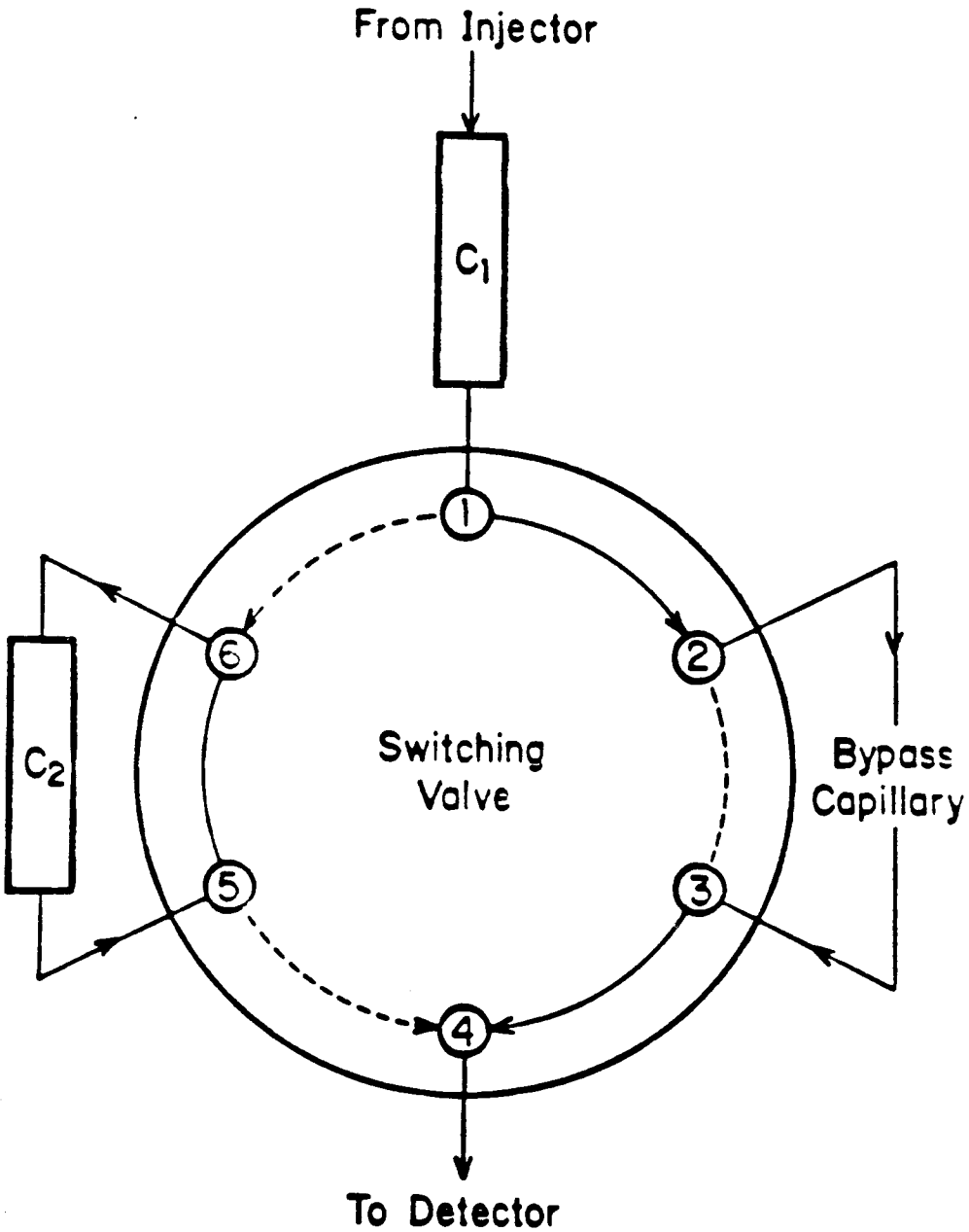


FIGURE 2: Chromatographic column and switching valve arrangement. Position I internal valve connections are designated by ____ and Position II by ----.

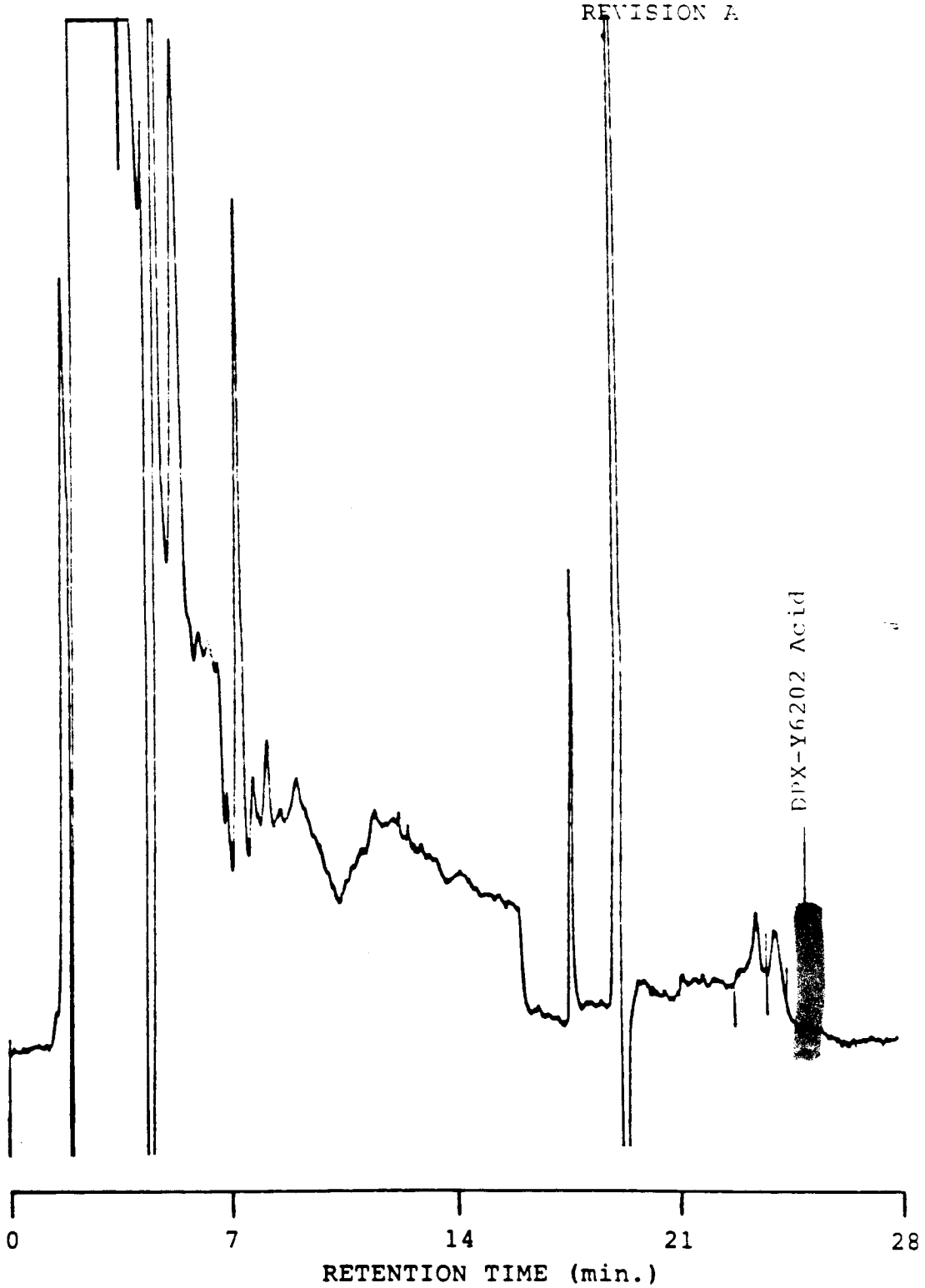


FIGURE 3: Chromatogram of a day 21 [redacted] sample from Cow No. 2.

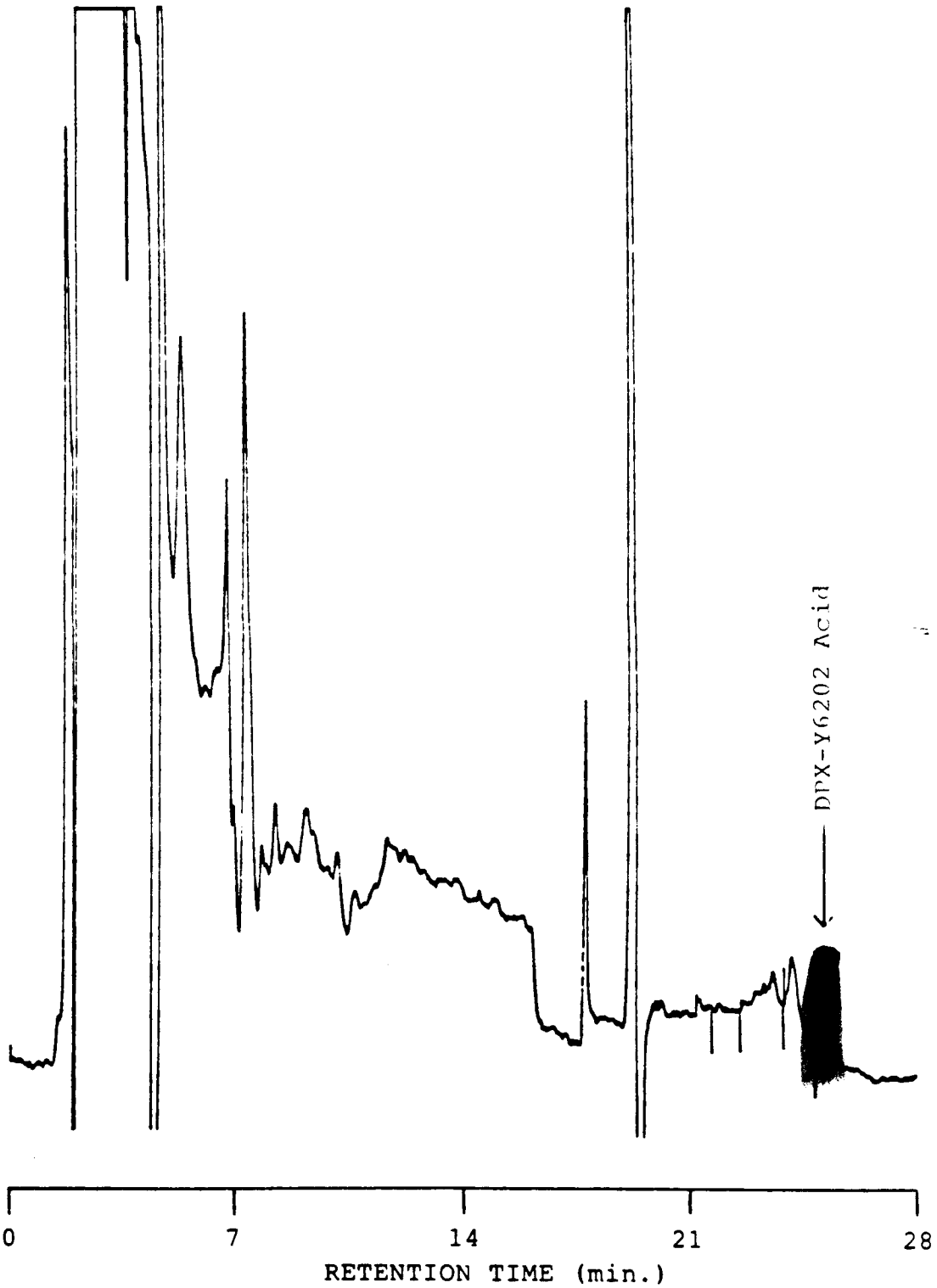


FIGURE 4: Chromatogram of the same control milk sample as in Figure 3 fortified with [redacted] ppm DPX-Y6202 (Recovery = 100%).

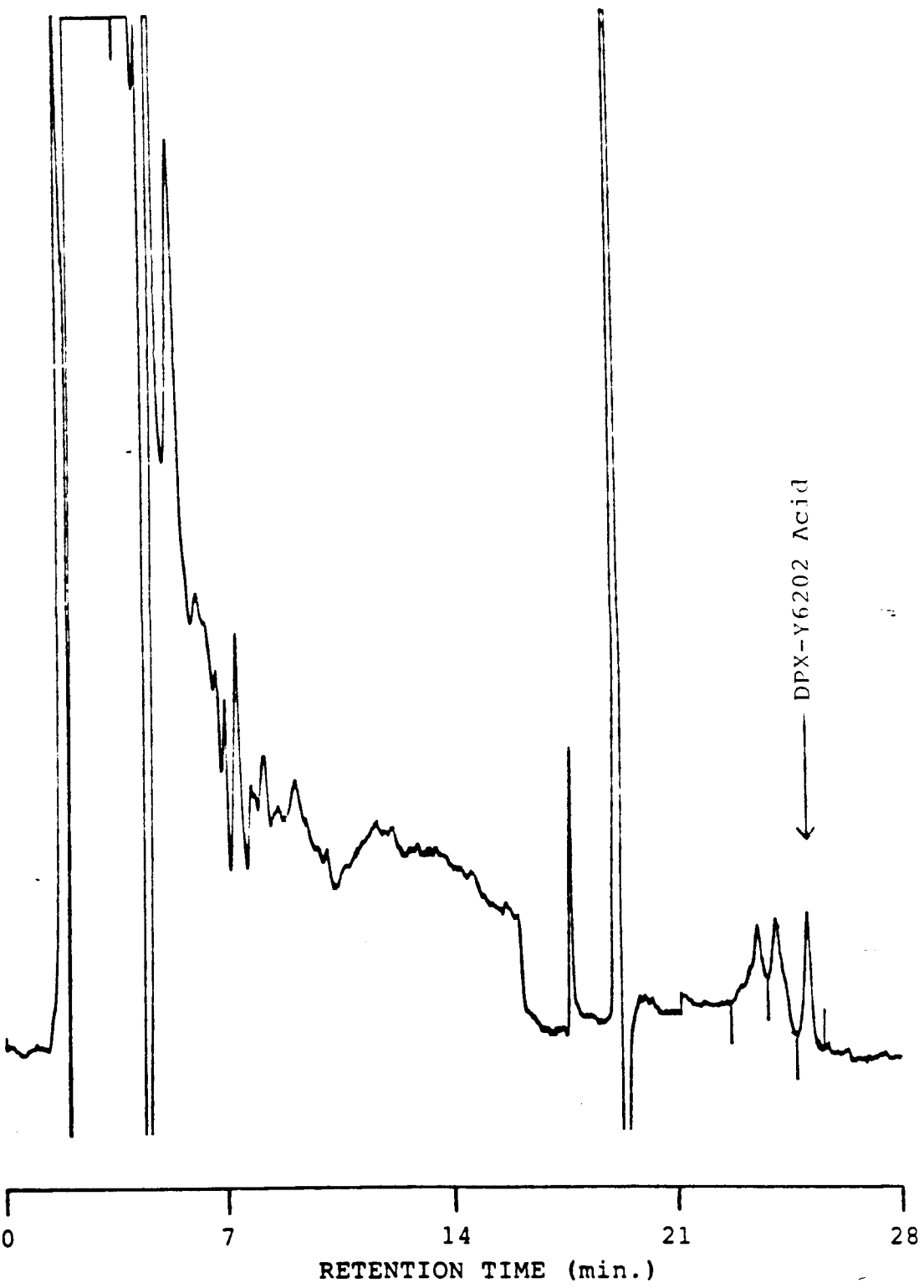


FIGURE 5: Chromatogram of a day 28 am milk sample from Cow No. 11 which was fed 5.0 ppm DPX-Y6202. The level of DPX-Y6202 Acid in the milk is 0.02 ppm.

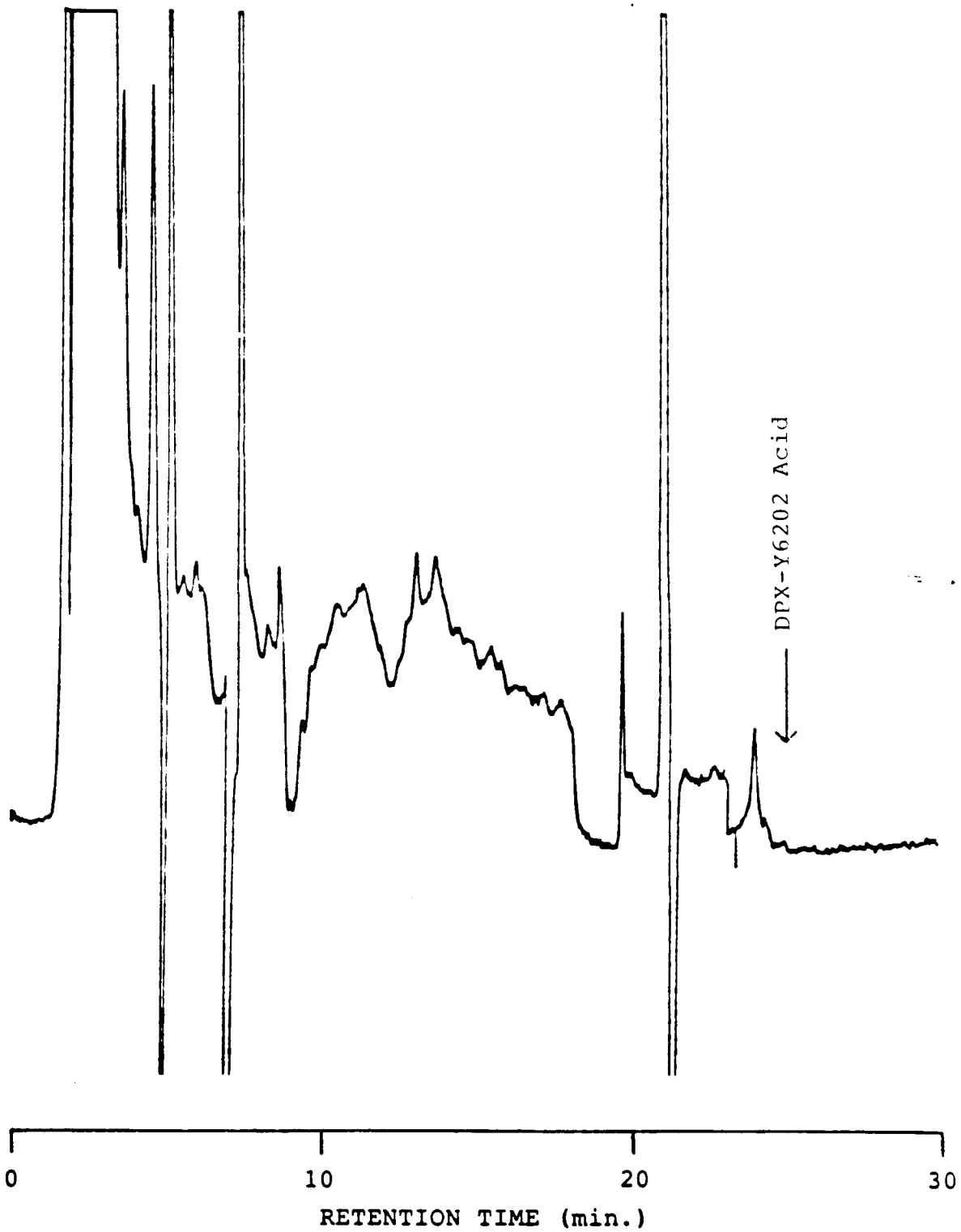


FIGURE 6: Chromatogram of a day 16 control skim milk sample from Cow No. 2.

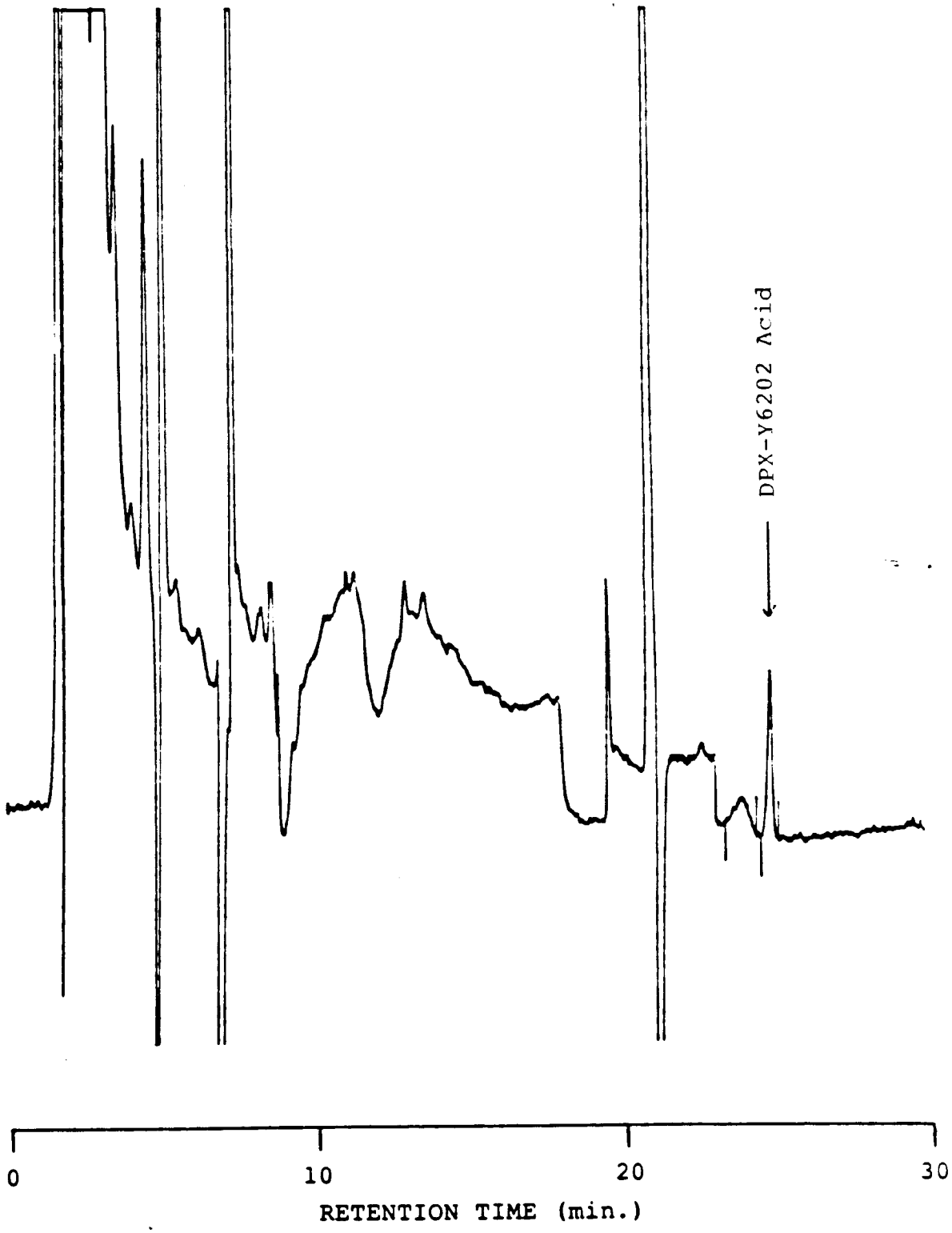


FIGURE 7: Chromatogram of the same control skim milk sample as in Figure 6 fortified with 0.02 ppm DPX-Y6202 Acid (Recovery = 85%).

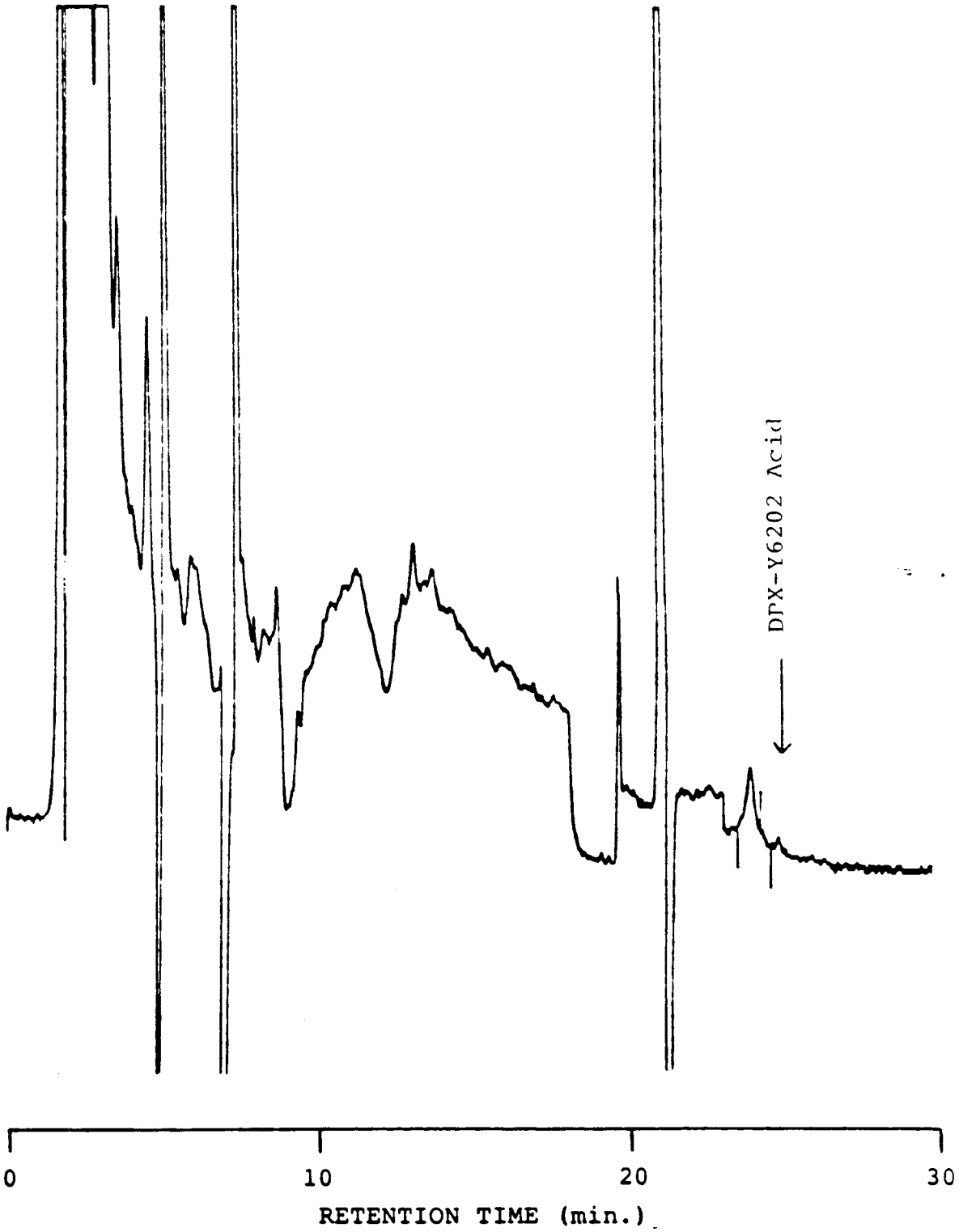


FIGURE 8: Chromatogram of a day 16 skim milk sample from Cow No. 11 which was fed 5.0 ppm DPX-Y6202. The level of DPX-Y6202 Acid is less than the detection limit of 0.01 ppm.

STORAGE LOCATION OF RAW DATA, REPORTS

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