

Study Title

DETERMINATION OF RESIDUES OF
DPX-Y5893 AND ITS METABOLITES IN CROPS,
ANIMAL PRODUCTS, SOIL AND WATER

Data Requirement

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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).

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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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SUMMARY/INTRODUCTIONSCOPE

Savey[®] Miticide is an experimental miticide used on a variety of crops such as apples and citrus. The active ingredient is trans-5-(4-chlor-phenyl)-cyclohexyl-4-methyl-2-oxo-3-triazolidinecarboxamide (DPX-Y5893). An analytical method is described for the determination of residues of DPX-Y589 and its metabolites in crops, animal tissues and products, soil, and water. A variety of crops, animal tissues, milk and eggs were tested during the course of method development, making the method suitable for a broad spectrum of sample types. Medium textured soil, obtained locally and potable tap water samples were included in method development to extend the scope of the method.

The method should be suitable for a wide variety of soil types, based on the totally interference-free background for the test soil, following clean-up. Since water sample contaminants vary greatly, depending on source, a simplified method was developed for residues in "clean" water in which no sample clean-up is required. As written, the method would include most tap water, spring water, well water, etc. (without sample clean-up). However, utilization of the silica column clean-up step described should extend the method to include all other water samples. The extraction and hydrolysis techniques used in the method were included in previous Company reports (ref. DU PONT STUDY NO. AMR-269-84, and Revisions 1, 2, and 3 of the same report).

PRINCIPLES OF METHOD

The compounds are extracted simultaneously from crop and tissue matrices by maceration in the presence of methanol, from soil by ultrasonic vibration in the presence of methanol, and from water by bulk phase partitioning with methylene chloride. Milk samples are extracted using successive methanol partitions and centrifugation. Following solvent concentration/conversion the parent compound and metabolites are converted by hydrolysis, to the more stable PT-1-3 metabolite. Water samples are analyzed by HPLC without clean-up, at a minimum quantifiable concentration (MQC) of about 0.1 ppb (calculated as parent). All other samples are cleaned up by liquid-solid chromatography using a small, disposable, silica gel column, then analyzed by HPLC at an MQC of about 20 ppb. Some samples, such as citrus, require an additional clean-up step in which the analyte is isolated from bulk interferences by gel permeation chromatography (GPC). Final qualitative and quantitative analysis of these samples are also performed by HPLC at an MQC of about 20 ppb.

COMPOUND IDENTITY

See Appendix.

MATERIALS/METHODSEQUIPMENT

(a) HPLC, DU PONT Model 850 equipped with a variable wavelength, UV detector (or equivalent instrumentation). *Required for all analyses.*

(b) HPLC column, 10cm x 4.6mm (ID), MICROSORB SHORT-ONE, C18, 3 micron (RAININ INSTRUMENT CO, Woburn, MA, 01801-4628), p/n 80-200. *Required for all analyses.*

(c) Solvent evaporator, Meyer N-EVAP (ORGANOMATION INC., South Berlin, MA 01549), p/n 11155-T. *Required for all analyses.*

(d) Multiport solvent valve, Baker MISER (J. T. BAKER CHEMICAL CO., Phillipsburg, NJ), p/n 7220-0. *Not required for most water analyses.*

(e) Sample homogenizer, Tekmar TISSUMIZER, probe and speed controller, Model SDT-1810, (THOMAS SCIENTIFIC, Swedesboro, NJ), p/nos. 3411-C10, 3411-C40, and 4311-C60. *Not required for milk, soil or water analyses.*

(f) Centrifuge, with capacity for 250 ml centrifuge bottles, SORVALL Model RC5C (DU PONT COMPANY, Clinical and Instrument Systems Division, Newtown, CT 06470). *Not required for water analyses.*

(g) Ultrasonic bath, 0.75 gallon capacity, Cole-Parmer, Model 8851 (Cole-Parmer Instrument Co., 7425 North Oak Park Ave, Chicago, IL 60648), p/n J-8851-00. *Required for soil analyses only.*

(h) Gel Permeation Chromatograph, GPC AUTOPREP, Model 1002A, (Analytical Bio Chemistry [ABC] Laboratories, Inc, P.O. Box

1097, Columbia, Mo 65205), p/n 981-001. *Required for citrus and some other complex matrices such as dehydrated crops.*

(i) Glass column for GPC, 600mm x 35mm, p/n 624-110 (ABC Labs), packed with BioBeads SX-3, 200/400 mesh, p/n 091-203 (ABC Labs). Pack column to maximum capacity (ie. 500-510 mm) with gel previously swelled in 50% cyclohexane/50% methylene chloride (v/v). *Required for citrus and some other complex matrices such as dehydrated crops.*

(j) Disposable clean-up columns. Use 4 ml reservoirs (p/n 600400), 20 μ m frits (p/n 640410), and SEPRALYTE 40 μ m silica gel packing (p/n 11012), all available from Analytichem International, 24201 Frampton Ave., Harbor City, Ca 90710. Slide a frit gently to bottom of each reservoir required for clean-up (one per sample). Weigh out 1.0 gram aliquots of the Si packing into 30 ml glass beakers. Pour a 1 gm aliquot of the packing through a small glass funnel, into each of the fritted reservoirs. Tap the bottom of each reservoir on a hard object a few times to compact the packing (the packing height will go from about 40mm to about 35mm). Slide another frit gently but firmly, to the top of the packing to complete the column preparation. The capacity of these columns results in very high efficiency and ruggedness for this method so that routine batch profiles are unnecessary. If, however, the columns are to be stored prior to analyses, they should be kept in air-tight plastic bags to prevent excess moisture accumulation on the Si surface. *Required for all sample analyses (except water).*

(k) pH meter, Corning Model 120 (Thomas Scientific, Swedesboro, NJ 08085-0099), p/n 4135-G10. *Required for all sample analyses (except water).*

(1) Sieve and receiver, No. 8, US Standard, 8 inch diameter, 2.36 mm (Thomas Scientific, Swedesboro, NJ 08085-0099), p/nos. 8323-R16 and 8328-S60. *Required for soil analyses only.*

REAGENTS AND STANDARDS

(a) Hexane, acetonitrile, ethyl acetate, methanol, methylene chloride and cyclohexane. Distilled-in-glass solvents from Burdick and Jackson Labs., Muskegon, MI.

(b) 0.1N sodium hydroxide solution. Dissolve 4.0 grams of sodium hydroxide in 1.0 liter of deionized water and mix thoroughly. Sodium hydroxide pellets, BAKER ANALYZED reagent (J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, NJ 08865), p/n 3722-1.

(c) Acidic water. Add concentrated hydrochloric acid, a drop at a time, with constant mixing, to 500 ml of deionized water contained in a one-liter beaker, until a pH of 2.0-2.1 is reached. About 15 drops will be required. Hydrochloric acid, 36.5-38.0%, BAKER ANALYZED reagent (J. T. Baker Chemical Co.), p/n 9530-1.

(d) Sodium chloride, crystal, BAKER ANALYZED reagent (J. T. Baker Chemical Co.), p/n 3624-1.

(e) DPX-Y5893 and PT-1-3, 99% purity, analytical standards - E. I. du Pont de Nemours and Co., Inc., Agricultural Products Department, Wilmington, DE 19898. Weigh out 100.0 mg of each compound into 30 ml beakers. Transfer the compounds to 100 ml volumetric flasks using small glass funnels. Rinse the beakers and funnels with several 10-ml aliquots of acetonitrile for a quantitative transfer to the flask. Swirl the contents of the flasks until the compounds have completely dissolved (add additional

acetonitrile if necessary). Add acetonitrile to the 100 ml mark, cap flasks and shake until well mixed. These stock standard solutions (1000 $\mu\text{g}/\text{ml}$ each compound) will be used for the preparation of all other standards and are stable for several months of intermittent use. Keep refrigerated between uses.

i. HPLC Working Standards - Transfer 10.0 mls of the PT-1-3 stock solution to a 100-ml volumetric flask, dilute to the mark with acetonitrile, cap and mix well for a 100 $\mu\text{g}/\text{ml}$ standard. Transfer 2.5 mls of the 100 $\mu\text{g}/\text{ml}$ solution to a 100-ml volumetric flask, dilute to the mark with acetonitrile, cap and mix well for a 2.5 $\mu\text{g}/\text{ml}$ standard. Transfer 1.0, 2.0 and 4.0 mls of the 2.5 $\mu\text{g}/\text{ml}$ standard to separate 100-ml volumetric flasks and bring the solvent to 25 mls in each by adding 24, 23 and 21 mls (respectively) of acetonitrile. Add 70 mls of deionized water to each flask, cap and mix. The mixing of these solvents (acetonitrile and water) will result in a temperature drop. Allow solutions to come to room temperature, add water to the mark, cap and mix well. The PT-1-3 HPLC standards thus prepared are at 0.025, 0.05 and 0.1 $\mu\text{g}/\text{ml}$ in a solvent matrix of 25% acetonitrile and 75% water. Using the HPLC conditions described later, the 0.1 $\mu\text{g}/\text{ml}$ standard should result in a peak height response of 60-70% of full scale with background noise of <1%, at a retention time of about 7 minutes. These standards are stable for several months of frequent use at room temperature.

ii. Spiking Standards - Transfer 10.0 mls of the DPX-Y5893 stock solution to a 100-ml volumetric flask, dilute to the mark with acetonitrile, cap and mix well for a 100 $\mu\text{g}/\text{ml}$ standard. Transfer 1.0 ml of the 100 $\mu\text{g}/\text{ml}$ standard to a 100-ml volumetric flask, dilute to the mark with acetonitrile, cap and mix well for a

1.0 µg/ml spiking standard. Use 1.0 ml of the 1 µg/ml standard for spiking a 50-gram sample at 0.02 ppm and increased volumes as required for higher concentration spikes. Additional standard dilutions may be required for spiking smaller sample weights or for spiking water samples. These standards are stable for several months of frequent use at room temperature.

iii. GPC Standard - Transfer 1.0 ml of the 100 µg/ml PT-1-3 standard (used to prepare HPLC standards) to a 100-ml volumetric flask and bring to the mark with a 1:1 (v/v) mixture of cyclohexane and methylene chloride. Cap and mix well for a 1.0 µg/ml GPC standard. This standard will be used to determine a profile of PT-1-3 by GPC for samples requiring this step. Keep this standard tightly capped and refrigerated between uses to prevent evaporation of methylene chloride. Discard after 3 months of storage.

ANALYTICAL PROCEDURE

(a) Sample preparation

1. Crops and animal tissues

Sample preparation will vary with crop type, sample size, and available equipment. In general, a 50-gram subsample will be taken for actual analysis which must be representative of entire sample received. Most samples will require thorough chopping with a knife or preferably, an industrial food processor, followed by thorough, manual mixing with a large spoon or spatula in order to form a homogenate. Watery crops require special care since they tend to separate into juice, peels, seeds, pulp, fibers, etc. Samples of this type must be mixed intermittently

during the course of any subsampling to insure that a representative sample is indeed obtained. Since peels may contain a large portion of total residue while being a small fraction of the total sample weight, it is especially important for the peels to be evenly distributed throughout the sample homogenate. If watery crop sample homogenates are to be frozen prior to subsampling, the entire sample homogenate must be thawed and remixed prior to any subsampling, since the samples probably separated into zones according to density. DO NOT attempt to subsample from partially thawed homogenates by sampling from a liquid layer or chipping off pieces of icy sample.

2. Soil

Fit the 8-inch, No. 8 sieve into the receiver and add soil slowly through the sieve until the receiver is full or until the entire sample has been added. Discard rocks and plant debris remaining on the sieve. If the soil is lumpy or wet, add small amounts of soil to the sieve and work it through the sieve using a metal or plastic spatula. Discard extraneous materials between each aliquot of soil. When the entire soil sample has been passed through the sieve, blend it into a homogeneous mixture by rolling and tumbling in a large, wide-mouth jar or plastic bag with sufficient headspace (usually 2-3 minutes is sufficient).

3. Muddy soil

If the samples are too wet to be manageable some drying may be required prior to sieving. Spread the samples out on aluminum foil to minimize the time required for drying. Over-night is usually sufficient for adequate drying. If the drying time is significant relative to sample treatment interval (for instance a

0-time interval), a sample should be spiked prior to drying. Weigh out a 50-gm subsample of the untreated soil on a piece of foil and spike with 0.5 ml of the 100 µg/ml DPX-Y5893 standard, distributing the standard over the surface area. Allow this sample to dry for the same time (and temperature) as the other samples but do not pass it through the sieve. After drying, pour the spiked sample into a 250-ml polyethylene centrifuge bottle and save the foil (spiked sample only) for rinsing at the extraction step. This 1.0 ppm spiked sample will be analyzed along with the other samples to monitor possible affects of the drying step.

4. Soil moisture determination

When samples have been sieved and thoroughly mixed, transfer to suitable, labeled containers such as wide-mouth jars. Using a spatula or spoon, transfer 10.0 grams of each sample (not spiked) to an aluminum foil weighing vessel or petri dish. Dry the samples in a drying oven at 100°C for 2 hours, allow to cool and re-weigh each to determine the moisture contents. The "% moisture" is the weight loss (in grams) multiplied by ten. Record the "% moisture" for later reporting or for calculation of residues on a "dry weight" basis.

(b) Extraction

1. Watery crops, animal tissues, and eggs

Weigh 50 grams of representative sample into a 250 ml polypropylene, widemouth, centrifuge bottle and add 100 ml of methanol. Insert the Tekmar homogenizer probe to about one-half the depth of the methanol and gradually increase speed until large pieces are macerated. Continue at steady, medium speed until mixture appears homogenous, but not longer than one minute. Shut

off motor and raise unit until bottom of shaft assembly is about one cm below top of bottle. Immediately rinse lower shaft and blade assembly with a few mls of methanol using a disposable pipet, allowing the rinses to drain into the bottle. It is not necessary to attempt to recover small particles remaining on the blade after rinsing.

Balance samples in preparation for centrifugation using methanol and centrifuge samples for five minutes at 5000 rpm. Decant methanol extract through fast-flow, fluted filter paper into a 250 ml graduated, mixing cylinder. Add 50 mls of fresh methanol to the solids in the centrifuge bottle, shake mixture vigorously until solids cake is completely broken up, balance and centrifuge as before. Decant methanol through filter paper, into mixing cylinder containing initial extract. Repeat solids rinse with 50 mls additional methanol, centrifuge and decant as before. Total extract volume should be slightly over 200 ml at this point. Stopper cylinder and mix by shaking. If the solution appears clear or only slightly cloudy adjust volume to 250.0 ml with methanol, stopper cylinder and mix extract thoroughly by shaking. If the solution appears cloudy or turbid, decant into a clean centrifuge bottle, balance with additional methanol and centrifuge at 5000 rpm for 5 minutes. Decant the extract into a 250-ml graduated mixing cylinder, adjust volume to 250.0 mls with methanol, stopper and mix by shaking. The sample-to-solvent ratio is 1.0 gram per 5.0 mls of solvent if 50 grams of sample were extracted. Proceed to "Acid/Base Washes" (Section (d)1.).

2. Low water content crops (dried nuts, grains, etc.).

To extract samples that are very low in % water, use the same method described above, except replace methanol with a mixture of 80% methanol/20% water(v/v) in each step that calls for methanol.

3. Soil

Transfer 50.0 grams of representative soil to a polyethylene centrifuge bottle using a spatula or small spoon. Add 100 mls of methanol and place in a sonic bath. Add water to the sonic bath until the water level is approximately the same as the solvent level in the sample containers. Sonicate the samples for 30 minutes. During the sonication, shake each sample vigorously (for about 5 seconds) at 5-7 minute intervals, to dislodge trapped air and refresh solvent around the soil particles. After sonication, balance the bottles with methanol and centrifuge for 5 minutes at 5000 to 7000 rpm. Decant the extracts into 250 ml graduated, mixing cylinders. Add 50 mls of fresh methanol to the samples, shake vigorously, repeat centrifugation and combine with original extract. Repeat rinse with an additional 50-ml aliquot of methanol, centrifuge and combine with original extract. Adjust final volume to 250 mls in mixing cylinders, stopper and mix thoroughly. The soil-to-solvent ratio is 1.0 gram per 5.0 mls of solvent if 50 grams of soil were extracted. Proceed to "ACID/BASE WASHES" (Section (d).1).

4. Water

Transfer a 200-ml aliquot of each water sample to 250-ml separatory funnels using 250-ml graduated cylinders. Add 20 mls of methylene chloride to each separatory funnel, stopper and shake vigorously for 30 seconds. Allow the phases to separate and

drain the lower phases into 40-ml graduated centrifuge tubes. Repeat the extraction using 20-ml aliquots of fresh methylene chloride and combine with initial extracts in centrifuge tubes. Discard the upper (aqueous) phase. The entire 40-ml extract will be used for subsequent analysis. Proceed to "HYDROLYSIS" (Section (d)2).

5. Milk

Weigh 50.0 grams of milk into 250-ml polypropylene centrifuge bottles, add 50 mls of methanol and 10 grams of sodium chloride. Cap the bottles and shake vigorously by hand for one minute. Balance the bottles using methanol and centrifuge at 5000 rpm for 5 minutes. Decant each extract into a clean centrifuge bottle. Add 70 mls of fresh methanol to the samples, cap and shake vigorously for 1 minute (be sure that the solids cake is completely broken up during the shaking process). Centrifuge samples as before and combine with initial extract. Add 70 mls of fresh methanol to the samples, shake and centrifuge as before. Combine this final extract with first two in centrifuge bottles, cap and shake to mix. The remaining sample cake may be discarded.

Balance extracts with methanol and centrifuge for at 5000 rpm for 5 minutes. Decant extracts (total of 220-230 mls) into 250-ml mixing cylinders, adjust volume to 250.0 mls with additional methanol, stopper and mix by shaking. The milk-to-solvent ratio is 1.0 gram per 5.0 mls of solvent if 50 grams of milk were extracted. Proceed to "Acid/Base Washes" (Section (d)1.).

(c) Fortifications

At least one spiked sample (fortification) must be

analyzed concurrently with each set of samples to monitor the integrity of the method. A spike at the MQL and the expected residue level is recommended. The following steps assume very low (or none) residues in treated samples.

1. All samples (except water)

During the course of weighing out samples in preparation for extraction, weigh out two 50-gram samples of the check (untreated) sample. Using a transfer pipet, distribute 1.0 ml of DPX-Y5893 spiking standard (1.0 $\mu\text{g}/\text{ml}$) over the surface of the sample contained in one of the centrifuge bottles. This results in a spiking level of 20 ppb. Leave the other check unspiked for analysis in order to determine background interference. Extract these samples along with treated samples.

2. Water

Transfer two 200-ml samples of untreated water to separatory funnels following the extraction procedure for water. Using a 500- μl syringe, transfer into one of the samples 200 μl of DPX-Y5893 spiking standard (1.0 $\mu\text{g}/\text{ml}$) while gently swirling the sample. This results in a spiking level of 1.0 ppb. Leave the other check unspiked for analysis in order to determine background interference. Extract these samples along with treated samples.

- (d) Derivative Formation

1. Acid/base washes

- i. Watery crops and soil

Pour 40.0 mls of extract (8.0 gram equivalent) into a 40-ml graduated centrifuge tube and concentrate to 9-10 mls using the solvent evaporation apparatus with the water

bath at 60°C. Add 15 mls of acidic water (pH=2) to the sample and mix by swirling. Pour the solution into a 60-ml separatory funnel and add 15 mls of methylene chloride (using it to rinse the centrifuge tube). Add 1 gram of sodium chloride to the mixture, stopper and shake vigorously for about 30 seconds. Allow the phases to separate and drain the methylene chloride (lower phase) into a clean 60-ml separatory funnel. Repeat the partition with 15 mls of fresh methylene chloride and drain into separatory funnel containing initial wash (30 mls total). Discard aqueous (upper) phase. Add 20 mls of 0.1N NaOH solution and 1 gram of NaCl to the separatory funnel containing the methylene chloride. Stopper and shake vigorously for about 30 seconds. Allow the phases to separate and drain the methylene chloride into a 40-ml centrifuge tube. Add 10 mls of fresh methylene chloride to the aqueous phase, repeat partition, and combine with initial methylene chloride wash (40 mls total). Proceed to "Hydrolysis" below.

ii. Animal products and oily crops

Pour 40.0 mls of extract (8 gram equivalent) into a 40-ml graduated centrifuge tube and concentrate to 20 mls using the solvent evaporation apparatus with the water bath at 60°C. Transfer the solution to a 125-ml separatory funnel and add 20 mls of acidic water (pH=2) containing 2 grams of sodium chloride. Add 20 mls of methylene chloride, stopper and shake gently with frequent venting. A moderate tumbling action for two minutes is efficient and produces the least emulsion. Drain the lower (organic) phase into a 40-ml centrifuge tube and add a second 20-ml aliquot of methylene chloride to the aqueous phase.

Repeat the partition and combine the organic phases in the centrifuge tube. Concentrate the methylene chloride to 0.5-1 ml using the water bath at 40°C, add 5 mls of hexane, mix and concentrate back to 0.5-1 ml. Repeat with a second 5-ml aliquot of hexane to remove all methylene chloride.

Add hexane to a volume of 20 mls, mix well and transfer to a 125-ml separatory funnel. Add 20 mls of hexane-saturated acetonitrile and shake with a moderate action for 2 minutes. Allow the phases to separate and drain the lower phase into a clean 40-ml centrifuge tube. Repeat partition with a second aliquot of hexane-saturated acetonitrile. Combine acetonitrile and discard hexane. Concentrate acetonitrile to 1 ml using the water bath at 60°C and add 10 mls of 0.1 N sodium hydroxide while acetonitrile is still hot (precipitates form on cooling). Replace the tube into the water bath (at 60 degrees), loosely stopper and allow to react for 30 minutes. Add water to the 40-ml mark and 3 grams of sodium chloride. Stopper and shake until salt is dissolved. Transfer aqueous solution to a 125-ml separatory funnel and add 40 mls of methylene chloride using it to rinse the centrifuge tube. Shake the mixture with moderate action (to avoid emulsions) for about 2 minutes and allow the phases to separate. Drain the lower (organic) layer into a 40-ml centrifuge tube and add 2 mls of methylene chloride to the separatory funnel, using it to rinse any emulsion that may remain. Combine the 2 mls with the original 40 mls and discard the aqueous layer. Proceed to "Disposable Si columns" (Section (e)3.).

2. Hydrolysis - All samples except animal products and oily crops

Concentrate methylene chloride to about 0.5 ml, using the solvent evaporation apparatus with the water bath at 40°C. Add 2 ml of acetonitrile and concentrate back to about 0.5 ml. Add 5 ml of 0.1 N NaOH, stopper loosely, place in a water bath at 60°C and allow to react for 30 minutes. Remove samples from water bath, add water to the 40-ml mark, cap and shake until mixed. Pour aqueous mixture into a 125-ml separatory funnel and add 1 gram of sodium sulfate and 20 mls of methylene chloride (using the methylene chloride to rinse the tube). Stopper and shake vigorously for about 30 seconds. Allow phases to separate and drain methylene chloride (lower phase) into a clean 40-ml centrifuge tube. Repeat the partition with a 20-ml aliquot of methylene chloride and combine with initial methylene chloride wash (40 mls total). Proceed to "Clean-up" (Section (e)).

(e) Clean-up

1. None - Water only

In many cases, water samples will not require any clean-up beyond extraction, hydrolysis and solvent conversion. Concentrate methylene chloride from previous step to about 0.5 ml using the solvent evaporation apparatus with the water bath at 40°C. Add 2 mls of acetonitrile and concentrate back to 0.5 mls. Add acetonitrile to the 1.0-ml mark and water to the 4.0-ml mark. This results in a final sample-to-solvent ratio of 50 grams/ml if 200 mls of water were extracted. Proceed to "HPLC Analysis" (Section (b)2.).

If water samples are not from "clean" sources

(ie. tap, well, spring, etc.) and interference is likely, proceed from hydrolysis step to "Disposable Si Columns" (Section (e)3.).

2. Gel permeation chromatography - Citrus (and as required)

While the following GPC technique has been found essential only for citrus analysis, its capability provides an ancillary clean-up for other complex matrices such as dried foods and feeds where background interference can vary widely from the fresh commodity. For samples other than citrus, proceed to "Disposable Si Columns" (below). If interferences occur at the HPLC retention time of PT-1-3 and are consistent throughout the samples, then the GPC step should be performed prior to the Si clean-up. Inconsistent or sporadic interferences indicate contamination other than sample matrix which may not be resolved by GPC.

i. Elution profile

Install the packed column described in section "Equipment" (i) on the GPC AUTOPREP and set mobile phase (1:1 methylene chloride/cyclohexane) flow to 5.0 mls/minute according to manufacturers instructions. This column should result in PT-1-3 eluting off in a 60-ml cut following a 190-ml wash. The profile should however, be confirmed or adjusted as follows: (1) set the instrument for 0 minutes "dump", 54 minutes "collect", and 0 minutes "wash", (2) inject 10 mls of the GPC standard into the 5-ml sample loop #1 and advance back to the #00 position, (3) place collector tube #1 in a 250-ml graduated cylinder and push the "AUTOSTART" button, (4) collect 170.0 mls in the cylinder and immediately transfer the collector tube to a labeled 15-ml graduated

tube, (5) collect 10.0 mls in the graduated tube and repeat with an additional 9 labeled tubes for a total of ten 10-ml cuts for profile determination, (6) concentrate the 10-ml cuts to 0.2 mls each, add 2 mls of acetonitrile and reconcentrate to 0.2 mls using the solvent evaporation apparatus at 60 degrees C, (7) add acetonitrile to make 1.0 ml each then water to a volume of 4.0 mls each, (8) analyze each fraction by HPLC using the same parameters as sample analysis, (9) determine the total volumes required for the dump and collect cycles and divide by 5 (mls/minute) for instrument time settings (no wash cycle is required), (10) use these settings for all subsequent sample analyses.

ii. Sample clean-up

Concentrate the 40-ml methylene chloride, sample matrix to 5.0 mls using the solvent evaporation apparatus with the water bath at 40°C. Add 5.0 mls of cyclohexane, stopper and mix thoroughly by shaking. Inject this entire sample into 5.0-ml GPC sample loop. After all samples have been injected according to this procedure, make sure the instrument settings are at: 38 minutes "dump", 12 minutes "collect" and 0 minutes "wash" (unless determined otherwise during previous profile step). Advance the sample loop selector to the #00 position. Place the numbered, sample collector tubes into labeled, 125-ml Erlenmeyer flasks and push the "AUTOSTART" button. When the clean-up sequence is complete, remove the Erlenmeyer flasks and concentrate the "collect" fractions to 20-30 mls using the solvent evaporation apparatus with the water bath at 50°C. Decant the solvent mixture into labeled, 40-ml, graduated centrifuge tubes for further concentration and

rinse the flasks with a few mls of methylene chloride. Proceed to next step "Disposable Si Columns".

3. Disposable Si columns - All samples (except water)

i. Solvent conversion

Concentrate methylene chloride from derivatization step or the methylene chloride/cyclohexane from the GPC step to 0.5-1 ml using the solvent evaporation apparatus with the water bath at 40°C. Add 5 mls of hexane using the hexane to rinse the walls of the 40-ml tubes and dissolve or suspend all solids (sonication may be necessary for some samples). Concentrate hexane back to 0.5-1 ml. Repeat this step with a second 5-ml aliquot of hexane to remove any traces of methylene chloride. Adjust final volume to 2 mls with hexane and dissolve or suspend any remaining solids.

ii. Column conditioning

Clean-up columns must be conditioned just prior to introduction of the sample matrix. Attach a 20-ml syringe to the top of the BAKER MISER multiport valve and one of the valve, solvent lines into a 250-ml Erlenmyer flask containing hexane. Affix one of the pre-packed columns (1 gram of silica gel) to the bottom of the valve and draw about 10 mls of hexane into the syringe. Force 7-8 mls of hexane through the column with enough pressure to expel all the air (voids) from the column. A well conditioned column should have a gelled look through-out the packing with no powdery appearance. Remove the column while there is still hexane remaining above the top of the frit. Go to the next step (sample introduction) before the column begins to go dry.

iii. Sample introduction

Using a disposable pipet, transfer the hexane sample matrix (2 mls) to the top of the prepared column in two or three small aliquots, forcing each aliquot onto the column packing using a rubber syringe bulb for pressure (or the multiport valve may be used for this purpose, using air pressure instead of solvent pressure). Use 1-2 mls of hexane to rinse the sample container and add this to the top of the column in the same manner. Leave about 5 mm of hexane remaining above the column frit. Fasten the column back to the bottom of the multiport valve and use the syringe to force an additional 10 mls of hexane through the column allowing about 5 mm of hexane to remain above the frit. Discard all hexane column rinses.

iv. Analyte separation

Temporarily remove the column containing the sample and place one of the solvent lines from the multiport valve into a 250-ml Erlenmyer flask containing 20% ethyl acetate/80% hexane (v/v). Using the syringe at the top of the multiport valve, rinse the syringe and valve with the new solvent, and fill the syringe with the solvent. Quickly replace the column containing the sample and force 15 mls of this mixture through the column. This first aliquot may be discarded. Force a second 15-ml aliquot through the column and save for analysis. Flow rate through the column should be in the range of 5-10 mls per minute. Discard the disposable, clean-up column.

INSTRUMENTATIONHPLC CONDITIONS

HPLC columns and operating parameters are chosen which achieve optimum balance between sensitivity and degree of resolution with symmetrical peaks emerging at reasonable retention times. Retention times are nominal since variation will occur between system configurations, column age and manufacturer, etc.

Instrument: DU PONT HPLC, Model 850.

Detector: DU PONT UV, Model 8800, variable wavelength, at 225 nm, 0.005 AUFS.

Column: C18, 3 micron, 4.6mm x 10cm, at 40°C

Mobile phase: Acetonitrile 40%, water 60%, isocratic, at 1.0 ml/min.

Injector: Manual 6-port valve with 100 µl sample loop.

Recorder: LINEAR Model 0156-0000, strip chart at 10 mv and 0.5 cm/min.

Typical response: 10 ng of PT-1-3 should give a response of about 60% FSD, with a noise level of <1% and a retention time of 7 minutes.

(b) Sample analysis

1. Solvent conversion

Concentrate the cleaned up sample extract in a 15-ml graduated centrifuge tube to 1 ml using the solvent evaporation apparatus at 60°C. Add 2 mls of acetonitrile and concentrate back to 1 ml to remove all hexane. Add 1 ml of acetonitrile and then water to the 8.0-ml mark. Stopper and shake to mix. The solution at this point will be 25% acetonitrile and 75% water containing one gram of sample per ml.

2. HPLC analysis

Inject cleaned up samples with intermittent injections of PT-1-3 standards. Construct a calibration curve by plotting $\mu\text{g/ml}$ of standard injected versus peak height (or area). Determine $\mu\text{g/ml}$ of PT-1-3 in samples from calibration curve and calculate ppm of Y5893 (+ metabolites) according to the following equation:

$$\frac{\mu\text{g/ml PT-1-3} \times 1.55}{\text{grams/ml sample}} = \text{ppm DPX-Y5983 (+ metabolites)}$$

where 1.55 is the conversion factor for PT-1-3 to DPX-Y5893.

INTERFERENCES

(a) Labware

Interfering peaks which may be encountered during HPLC analysis are generally the result of exposure of solvents to soft plastic or rubber, such as pipette bulbs, plastic tubing, rubber stoppers. Care should be taken to limit solvent exposure only to inert materials such as glass, metal or inert plastics (linear

polyethylene, fluorocarbons, etc.). Since this can be a random and nonreproducible occurrence, reagent blanks may fail to detect such interferences.

(b) Sample matrices

Interfering peaks developed in cleaned-up sample extracts which were allowed to sit at room temperature for extended periods (approximately 48 hours) and in samples sitting in a hot water bath beyond the required time. It is recommended therefore, to keep samples/extracts frozen or at least refrigerated, while not in actual use and to keep hot water bath time to a minimum to prevent decomposition of sample matrices.

CONFIRMATORY TECHNIQUE

A qualitative analysis can be performed using the partition coefficient for PT-1-3 in hexane/water. Concentrate the sample matrix left after HPLC analysis to exactly 1/2 volume using the solvent concentration apparatus at 60°C. This step removes all acetonitrile and doubles the analyte concentration. Inject an aliquot of the water solution into the HPLC. Add a volume of hexane equal to the volume of remaining water, shake vigorously and allow to separate. Remove the hexane and reanalyze the water. Duplicate this procedure using 8.0 mls of an HPLC standard similar in concentration to the apparent PT-1-3 in the sample. This partition step should result in 38% \pm 4% PT-1-3 remaining in the water phase, however, use the HPLC standard results for final comparison. A deviation of greater than 4% indicates that the peak in question is probably not pure analyte.

APPROXIMATE TIME REQUIRED FOR ANALYSIS

A "set" of samples is a group of samples which are carried through extraction, clean-up and quantitation together. A typical set of samples would include one untreated sample, one or two spikes and two to eight treated samples. A set of water samples will require 1 day. A set of watery crop, animal tissue, milk, eggs, or soil samples will require 2 days. A set of citrus samples will require 3 days (where "days" means bench-man-days). Time estimates include sample processing and manual HPLC injections. Time estimates exclude instrument and apparatus set-up, house-keeping, and bookkeeping.

WATERY CROPS - USE DEFINITION

Watery crops are generally considered those having a nominal water content of about 70% or greater. The tables giving %-water in the Pesticide Analytical Manual (PAM) Vol. 1, Section 202 are recommended when the water content of samples is unknown. This can be an aid in deciding whether or not the GPC step may be necessary. Watery crops are assumed to mean those in the fresh, succulent state. If samples are field-dried or processed to remove water, they should not be considered as watery samples regardless of the nominal water content of the fresh commodity.

RESULTS AND DISCUSSIONACCURACY

Accuracy of the method appears to be generally consistent

for all sample types with an expected mean value of about 87% recovery from spiked samples. Recoveries ranged (all samples) from about 70% to 120%. Spiking levels ranged from 0.20 ppb to 1.0 ppb for water samples and from 0.008 ppm to 1.0 ppm for all other samples.

PRECISION

The standard deviation for this method was calculated to be 12.2%. The total population of recovery values was used to determine the standard deviation. The population parameter 'n-1' was used for the calculation. This standard deviation results in a variance of 14%.

LIMITS OF DETECTION AND QUANTITATION

The limits of detection and quantitation for this method were based on a detector (UV) response for the analyte which was at least twice the background noise level. Generally, the signal-to-noise (s/n) level was about 10/1. Noise here, does not include low level interferences from sample matrices, but photo/electrical noise which can vary with UV source age, line voltage fluctuations, etc., and noise due to mobile phase fluctuations. With time and use, s/n can vary somewhat as compound response (sensitivity) varies with the condition of the HPLC column. The following definitions for detectable levels will therefore assume an acceptable s/n which may range from the minimal 2/1 to about 10/1, to allow for variations from instrument to instrument and within the same instrument with time and use.

(a) Limit of detection (LOD)

Herein defined as the amount (in nanograms) of analyte which results in a recorder response that is twice the noise level. For PT-1-3, the LOD should be about 0.4 ng giving a 2% full scale response with a 1% noise level (ie. $s/n=2/1$). This is equivalent to an LOD for DPX-Y5893 of 0.6 ng using the conversion factor of 1.55.

(b) Minimum quantifiable concentration (MQC)

Herein defined as the practical, lower-limit of concentration achievable by a given method. (Where "practical" implies sufficient tolerance/ruggedness built into the method to allow for experimental variations.) The MQC for DPX-Y5893 in most commodities is 20 ppb, and 0.1 ppb in water.

LIMITATIONS

This method was designed with relatively broad tolerances to allow for column packing variations, sample matrix effects, volumetric variations, etc. While it is assumed that some material changes can be made to take advantage of existing equipment, certain steps must be strictly followed for successful analysis.

(a) Si clean-up columns

While there is a wide variety of disposable clean-up columns available from several vendors, there are none available at this writing which will serve as substitutes for the column described. The 1 gram of packing and column dimensions are essential for analyte isolation in most cases.

(b) Pressure vs vacuum

Solvent delivery to Si clean-up column must be via

pressure as stated in the method. A vacuum manifold, while faster, causes voiding in much of the column resulting in irreproducibility.

(c) Integrator

Use of an integrator should be avoided for calculations of residues in the area of the MQC, unless the s/n is at least 5/1. A strip-chart recorder, using peak-height measurements is excellent for low level results. A baseline drawn through the top of the noise and measurement to the top of the peak is rapid and a good approximation of peak height at very low levels.

CONCLUSION

The residue method as described, will work for a wide variety of sample types.

The extraction and derivatization portions of the method were previously developed and validated by other workers (see references Sec. I. A.) and remain basically unchanged. Much effort has gone into the development of rapid clean-up for a variety of sample matrices to extend the scope and usefulness of the method. The success of the method depends heavily on the proper use of 1-gram, hand-packed, disposable, Si clean-up columns, for which materials are readily available. These columns can easily handle the recommended equivalent sample weight (8 grams) without overloading or causing profile changes for the analyte. Most sample types will not require the GPC step. Interfering peaks at the HPLC

stage of analysis are usually due to soft plastics or decomposition of unrefrigerated sample matrix. Solvent volumes, partition coefficients, column profiles etc., were selected which both minimize solvent consumption (and disposal) and maximize tolerances for method ruggedness.

APPENDIX

Compound Identity

Typical HPLC Calibration Curve

Typical Chromatograms - Watery Crop (Grapes

Typical Chromatograms - Citrus

Typical Chromatograms - Soil

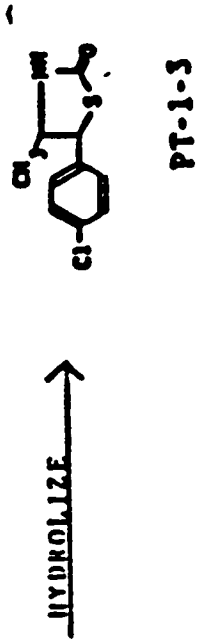
Typical Chromatograms - Water

Typical Chromatograms - Milk

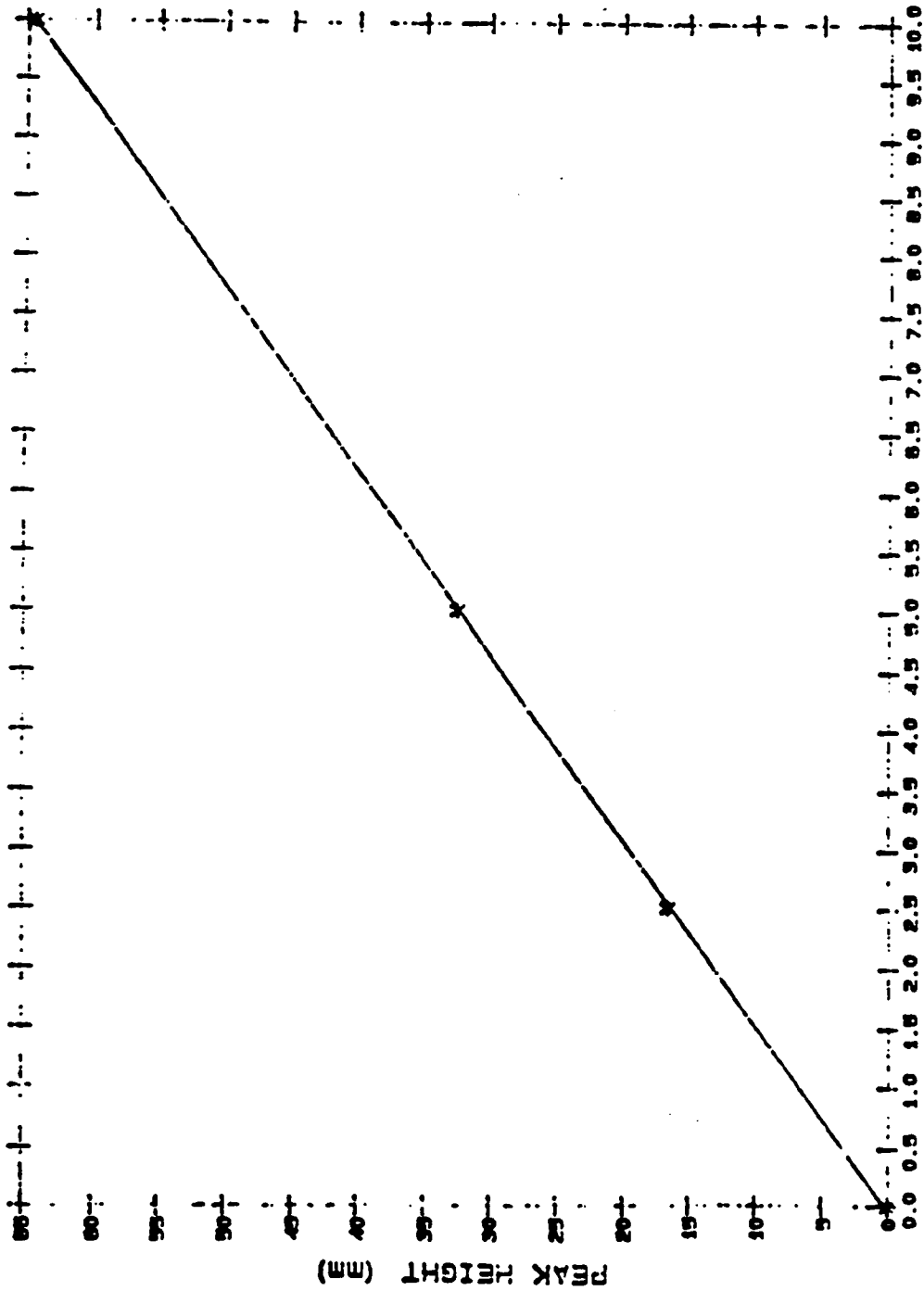
Typical Chromatograms - Cow Liver and Whole Eggs

Method Flowchart

Symbol ^o	Structural formula
NA-73 (trans) DPX-Y5893	
PT-1-2	
(cis-1) PT-1-4 (cis-2) (trans-1) (trans-2)	
PT-1-5	
(cis-1) PT-1-6 (cis-2) (trans-1) (trans-2)	
PT-1-7	
(cis) PT-1-8 (trans)	
PT-1-9	

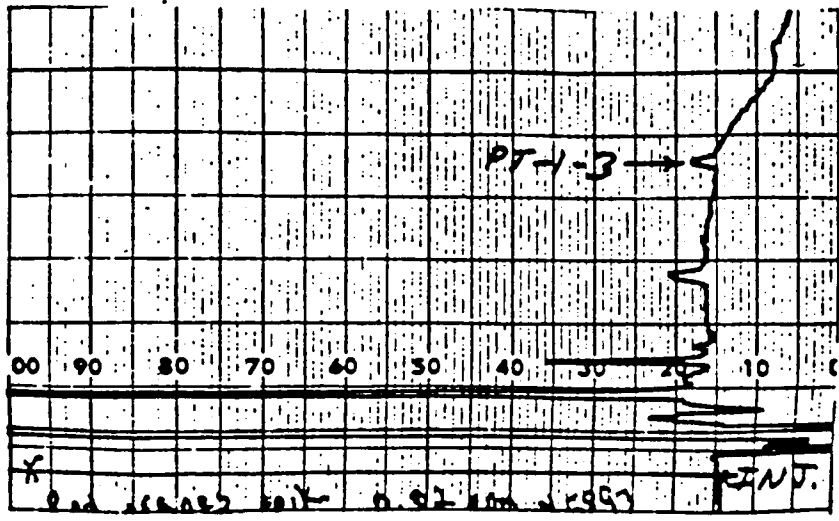


CALIBRATION CURVE

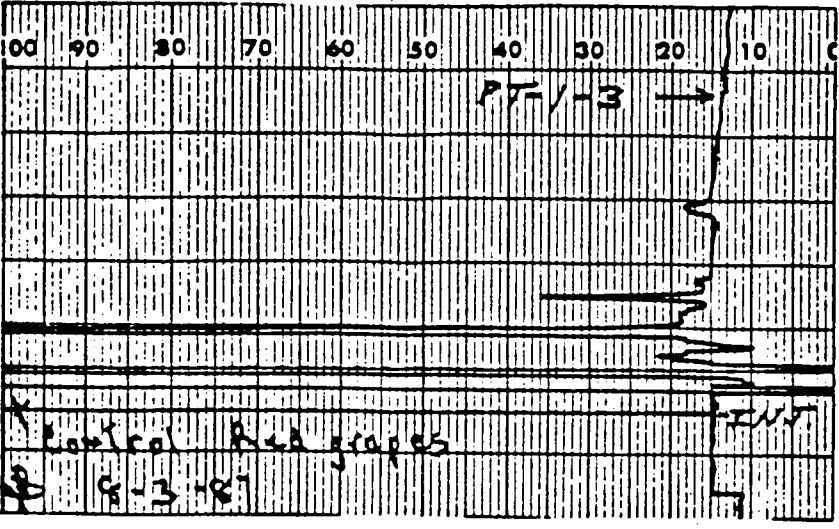


x PT-1-3/100ul
— 6.44mX + 0.2

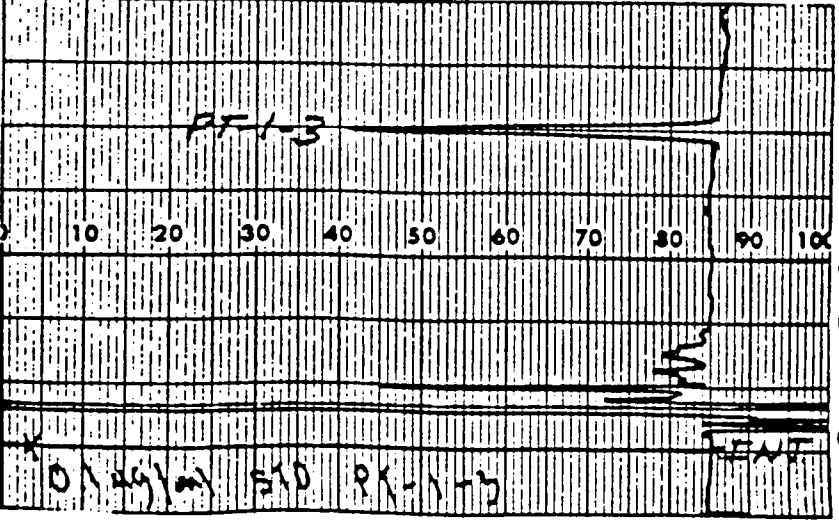
PT-1-3 (ng.)



CONTROL SAMPLE
SPIKED AT 0.02 PPM
DPX-Y5893
100. MG INJECTED

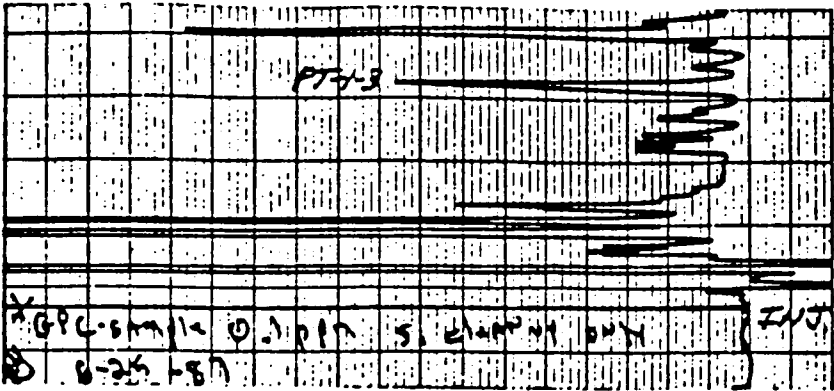


CONTROL SAMPLE
100. MG INJECTED

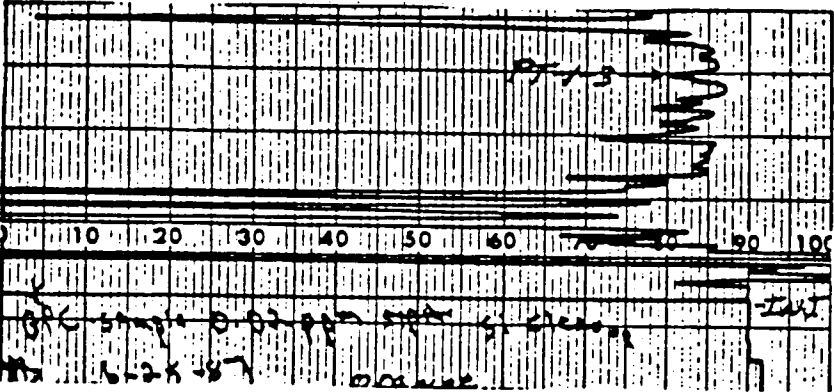


PT-1-3 STANDARD
10.0 MG INJECTED
(15.5 MG DPX-Y5893)

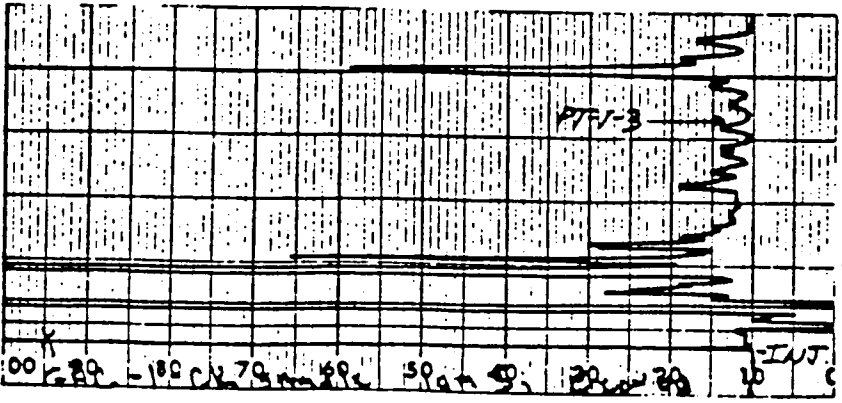
TYPICAL HPLC CHROMATOGRAMS OF RED GRAPE SAMPLES



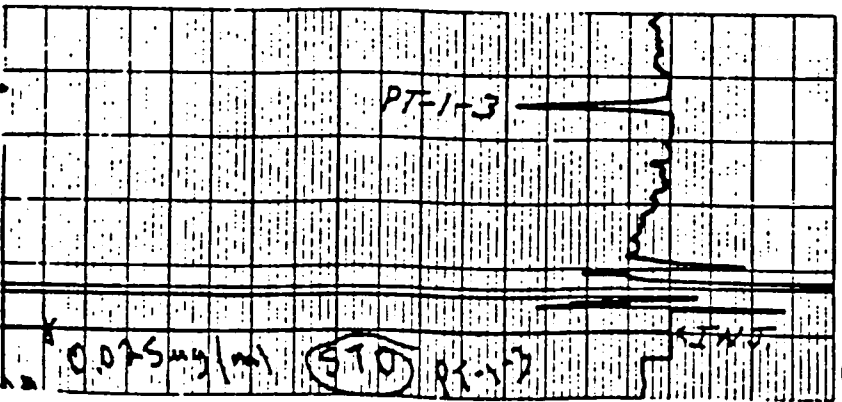
CONTROL SAMPLE
SPIKED AT 0.10 PPM
DPX-Y5893
100. mg/INJECTED



CONTROL SAMPLE
SPIKED AT 0.02 PPM
DPX-Y5893
100. mg INJECTED



CONTROL SAMPLE
100. mg INJECTED



PT-1-3 STANDARD
2.5 mg INJECTED
(3.88 mg DPX-Y5893)

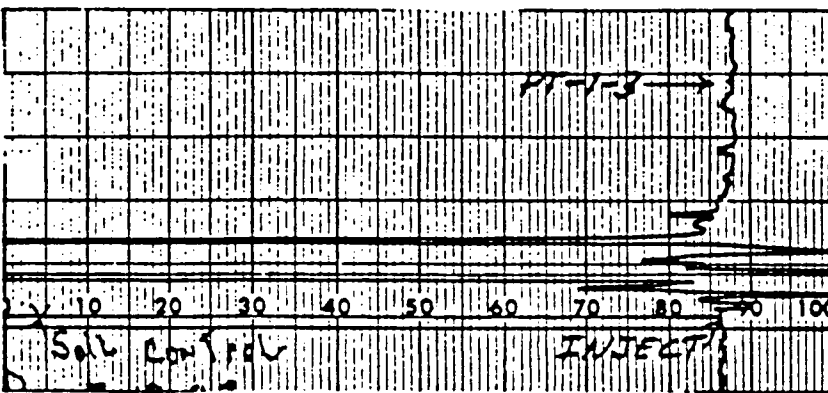
TYPICAL HPLC CHROMATOGRAMS OF WHOLE ORANGE SAMPLES



CONTROL SAMPLE
SPIKED AT 0.10
PPM DPX-Y5893
100. MG INJECTED



CONTROL SAMPLE
SPIKED AT 0.02 PPM
DPX-Y5893
100. MG INJECTED

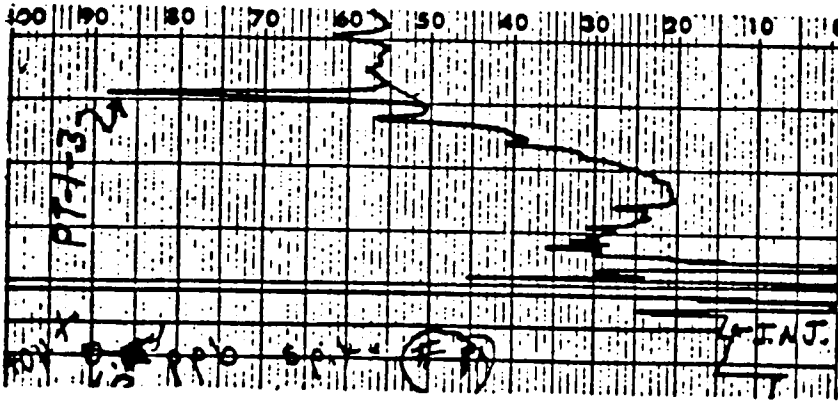


CONTROL SAMPLE
100. MG INJECTED

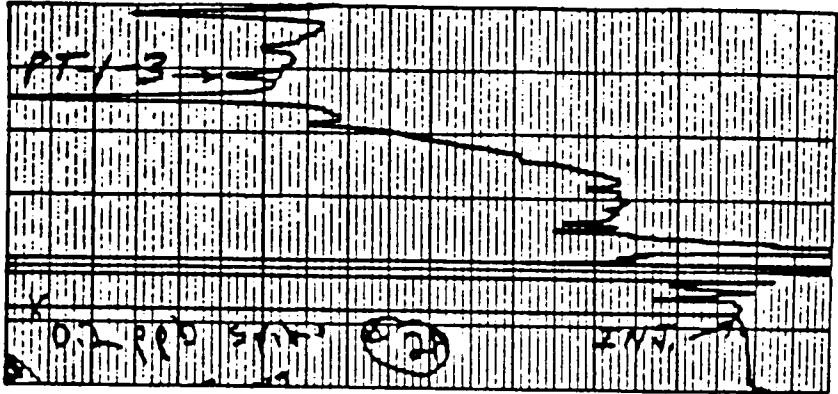


PT-1-3 STANDARD
5.0 MG INJECTED
(7.75 MG DPX-Y5893)

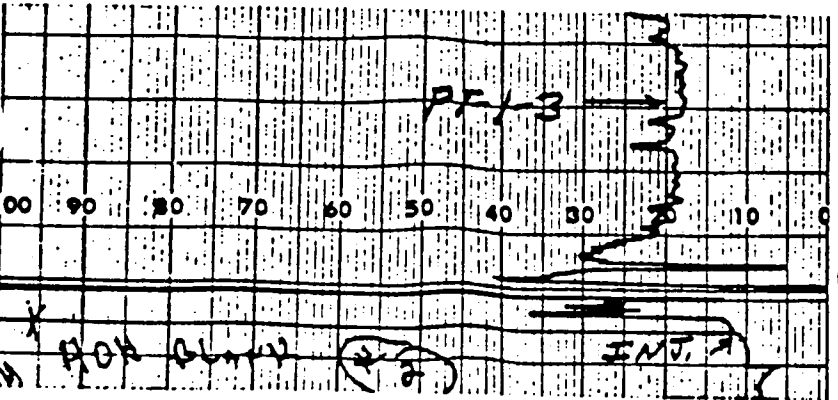
TYPICAL HPLC CHROMATOGRAMS OF SOIL SAMPLES



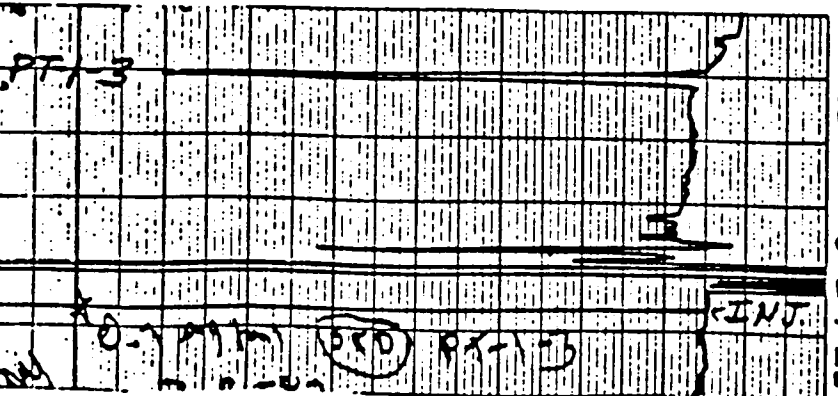
CONTROL SAMPLE
SPIKED AT 1.0 PPB
DPX-Y5893
10.0 g INJECTED



CONTROL SAMPLE
SPIKED AT 0.2 PPB
DPX-Y5893
10.0 g INJECTED

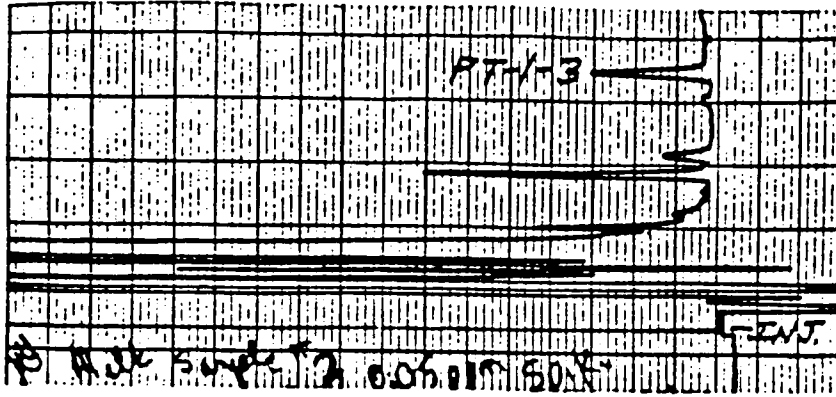


CONTROL SAMPLE
10.0 g INJECTED

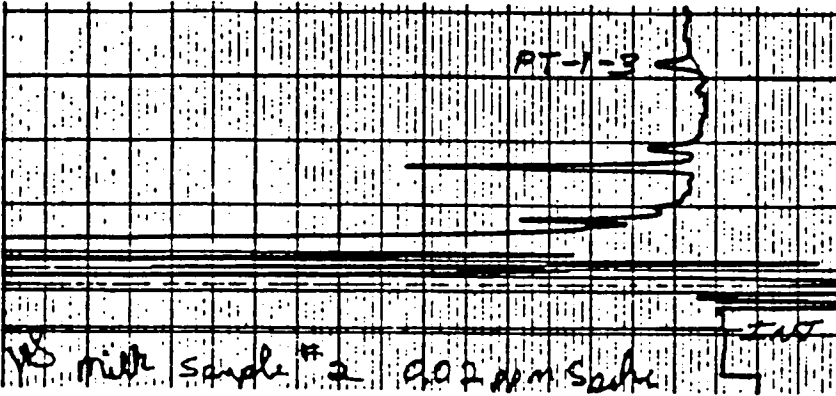


PT-1-3 STANDARD
10.0 mg INJECTED
(15.5 mg DPX-Y5893)

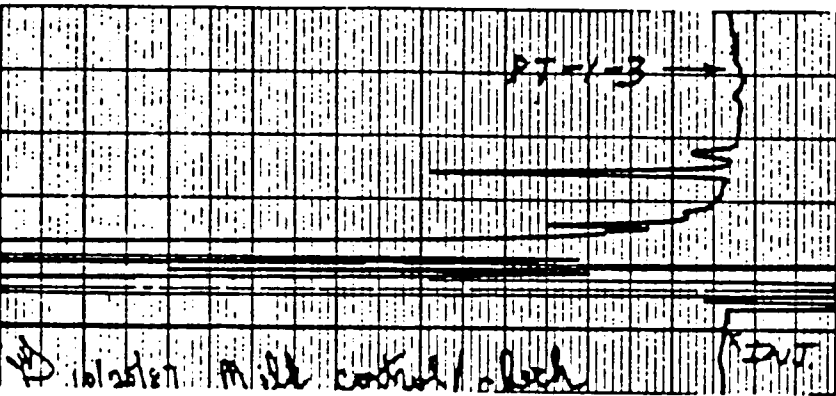
TYPICAL HPLC CHROMATOGRAMS OF WATER SAMPLES (NO CLEAN UP)



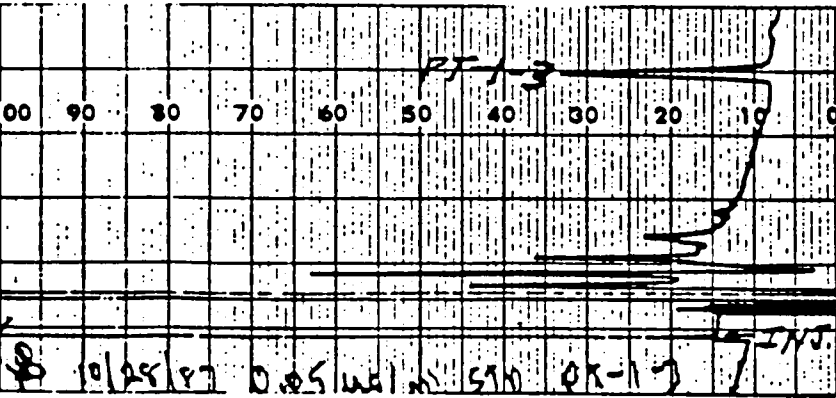
CONTROL SAMPLE
SPIKED AT 0.05 PM
DPX-Y5893
100. MG INJECTED



CONTROL SAMPLE
SPIKED AT 0.02 PM
DPX-Y5893
100. MG INJECTED

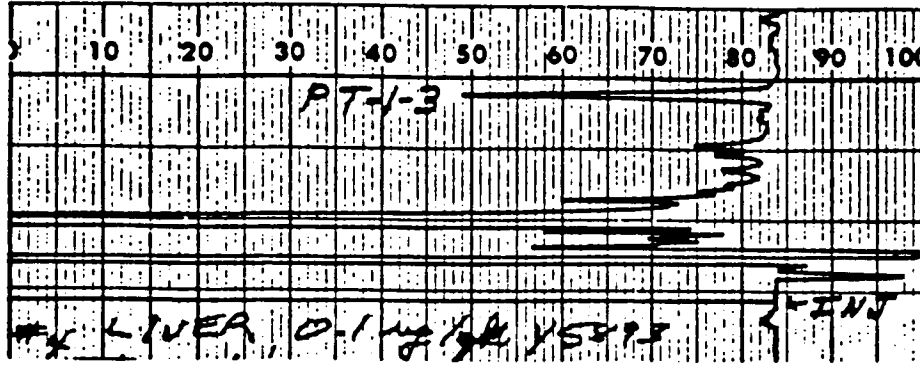


CONTROL SAMPLE
100. MG INJECTED

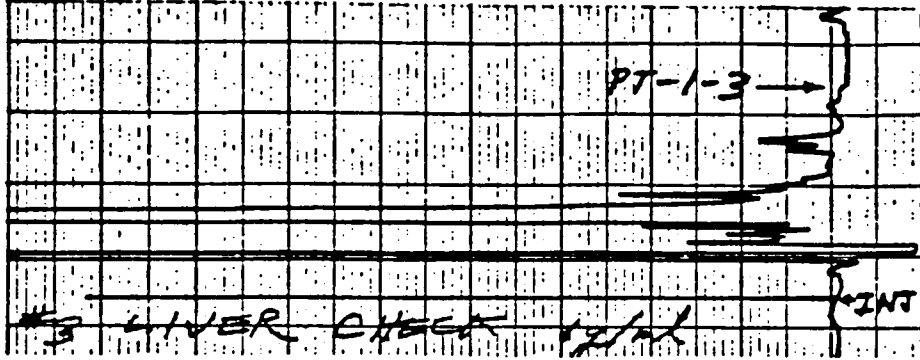


PT-1-3 STANDARD
5.0 MG INJECTED
(7.75 MG DPX-Y5893)

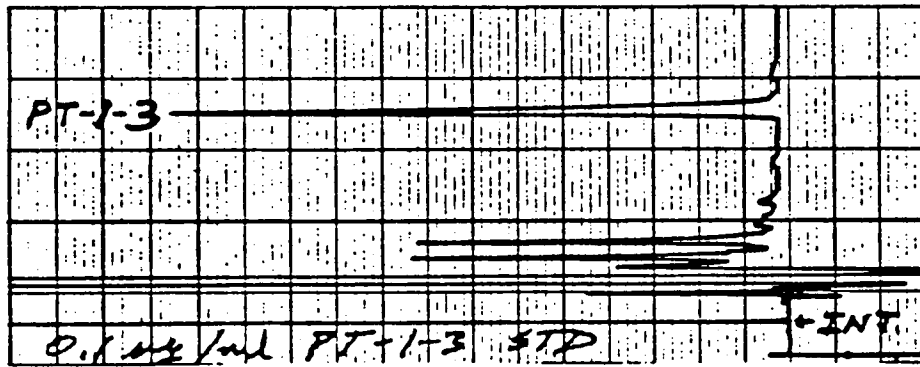
TYPICAL HPLC CHROMATOGRAMS OF WHOLE MILK SAMPLES



CONTROL LIVER
SPIKED AT 0.10 PM
DPX-Y5893
100 mg INJECTED



CONTROL LIVER
100 mg INJECTED



PT-1-3 STANDARD
10.0 mg INJECTED
(5.5 mg DPX-Y5893)

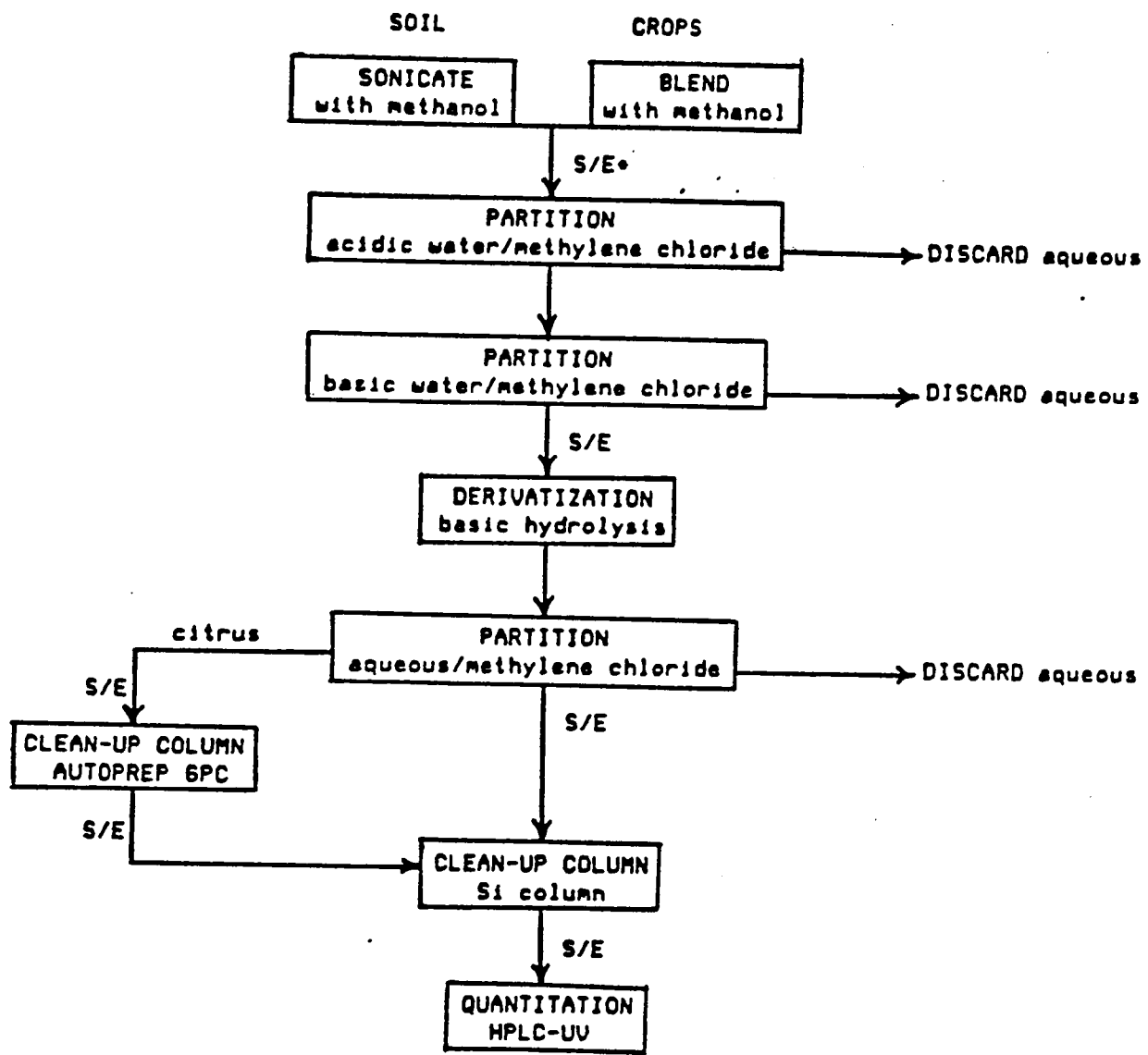


CONTROL EGGS
SPIKED AT 0.10 PM
DPX-Y5893
100 mg INJECTION



CONTROL EGGS
100 mg INJECTED

TYPICAL HPLC CHROMATOGRAMS OF EGGS AND COW LIVER



• SOLVENT EXCHANGE

METHOD FLOWCHART

STORAGE LOCATION OF RAW DATA, REPORTS

E. I. du Pont de Nemours and Company, Inc.
Agricultural Products Department
Experimental Station
Residue Studies Groups' Archives
Wilmington, Delaware 19898