CHAPTER III

EXPERIMENTAL

1. Apparatus

A Hewlett Packard HP 5890 gas chromatograph equipped with Flame Ionization Detector (FID) and Hewlett Packard HP3396 A Integrator.

A Hewlett Packard HP 5890 gas chromatograph equipped with Electron Capture Detector (ECD) and Hewlett Packard HP 3396 A Integrator.

A Hewlett Packard 25 m x 0.32 mm ID x 0.52 μm film thickness Ultra-1 (Crosslinked Methyl Silicone Gum) capillary column.

An Orion Research Digital Ion Analyzer model 710 A pH/ISE meter, USA.

A 40/35 HPLC Vacuum Filter for Solvents set with xx 1004703 clamp, Millipore, USA [-Graduated Funnel, -Disk Base, -1 liter Suction Flask]

A Waters pump, model DOA-U130-BN with pressure regulator, Millipore, USA.

Automatic Preparative Ultracentrifuge 55P-72 Hitachi, Japan.

Minicolumn for clean up step, 5.5 cm x 1.8 cm ID (the top part), 15.0 cm x 0.8 cm ID (the middle part), 9.5 cm x 0.1 cm ID (the end), Willers Laborbedarf Munster, Germany.

Glass fiber prefilter dimension 10 mm., Millipore, USA.

Graded density Glass microfiber filters, Multigrade GMF 150, 1 μm, 47 mm, Whatman, England.

An Alba watch, sw01-x001, Japan.

Banch Centrifuge, BTL.

Test tube 25 x 200 mm, pyrex, USA.

Empore™ Extraction Disks (Bakerbond Extraction Disks) with bakerbond C_{18}, 47 mm ID, Production of 3M, Distributed by J.T.Baker Inc.,USA.

Empore™ Filter Aid 400, High Density Glass Beads, 3M, USA.

Microsyringes 10.00, 50.00 and 250.00 μL Hamilton Company, USA.

Graduated pipettes 1.00, 2.00 and 5.00 mL

Volumetric pipettes 1.0, 5.0 and 10.0 mL

Volumetric flasks 5.00, 10.00, 25.00, 100.00, 250.00 and 1,000.00 mL

Beaker 5, 10, 25, 50, 150, 250, and 1,000 mL

All glass apparatus was washed in detergent, throughly rinsed with double distilled water and then soaked in dilute HNO_3 (1:1) overnight. The glass apparatus was then rinsed with double distilled water and baked in an oven at 150 °C for at least 3 hours, except volumetric flasks and pipettes. In the last step, all glass apparatus was double rinsed with elution solvents.

2. Chemicals

2.1 The Standard of Phthalate Ester (PEs)

Dimethylphthalate (DMP), Diethylphthalate (DEP), Di-n-butyl phthalate (DBP), Butylbenzylphthalate (BBP), Di(2-ethylhexyl)phthalate (DEHP) and Di-n-octylphthalate (DOP) which had percent purify of 99.0, 99.0, 99.0, 98.5, 99.5 and 95.5 respectively from Chem Service, Inc., West Chester, PA., USA.

2.2 Organic Solvents

Absolute methanol, hexane, ethyl acetate, ethanol and acetone from J.T. Baker Chemical Company, Deventer, Holland. All solvents were analytical

grade (AR Grade) and they were purified by fraction distillation in all glass apparatus and the distilled was checked for purify by gas chromatograph prior to use.

2.3 Reagents

Nitric acid, acetic acid and hydrochloric acid were analytical grade from E.Merck, Darmstadt, Germany.

Anhydrous Sodium sulphate, Sodium hydroxide and Sodium acetate were laboratory grade from E.Merck, Darmstadt, Germany.

Anhydrous sodium sulphate, sodium acetate, sodium hydroxide had to reduce background phthalate levels by heat 4 hours at 400°C.

Florisil pesticide grade (60/100 mesh) from Mallimlerodt Chemical, Parwas, France. Florisil might be contaminated with phthalate ester. Heating at 320°C and rinsing with elution solvent were recommended. To prepare for use, place 100.0 g of Florisil into a 500 mL beaker and heat for approximately 16 hours at 40°C. After heating, transfer to a 500 mL reagent bottle. Tightly seal and cool to room temperature. When it cooled, add 3.00 mL of double distilled water. Mix throughly by shaking or rolling for 10 min and let it stand for at least 2 hours. Store in the dark glass containers with ground glass stopper or foil-fined screw caps.

2.4 Standard Milk

The Standard milk that was processed like the sample milk but it was collected before packing and was obtained directly from milk center, Royal Chitlada Project.

3. Preparation of the Standard Solutions

3.1 The Stock Standard Solution of PEs in Methanol and Ethyl acetate

The 2,000.00 ppm stock standard solutions of each phthalate ester (PEs), i.e., DMP, DEP, DBP, BBP, DEHP and DOP were prepared by dissolving 0.1000 g of each standard and diluting it to the mark with the ethyl acetate in 50.00 mL volumetric flasks. The 200.00 ppm of standard solution of mixture phthalate esters was prepared by pipetting 5.00 mL of each 2,000.00 ppm stock standard solution into 50.00 mL volumetric flask and then diluting it to the mark with ethyl acetate.

The standard solution of PEs for preparation of the calibration curve, were prepared from this 200.00 ppm of the standard solution of PEs in ethyl acetate, by dilution of this 200.00 ppm standard solution.

The 5,000.00 ppm stock standard solutions of each phthalate ester in methanol were prepared by dissolving 0.0500 g of each standard and diluting it to the mark with the methanol in 10.00 mL volumetric flasks. The 250.00 ppm of standard solution of mixture phthalate esters was prepared by pipetting 5.00 mL each 5,000.00 ppm stock standard solution into 100.00 mL volumetric flask and then diluting it to the mark with methanol.

3.2 The pH Adjustment Solutions

A 1.0×10^{-2} M hydrochloric acid and a 1.0×10^{-2} M sodium hydroxide solution were employed for pH adjustment of the solution.

4. The Gas Chromatographic Condition

The previous GC conditions (73) were slightly modified in order to obtain suitable conditions for this study.

All of the standard solutions, spiked standard solutions and sample solutions, were injected 1 μL into GC under the conditions in Table 3.1 and 3.2. The Chromatogram of standard mixture solution of PEs on GC-FID and GC-ECD were shown in Figure 4.1 and 4.2.

Table 3.1 The gas chromatographic conditions for the study by GC-FID

GC Parameter	GC Condition
Analytical Column	25 m x 0.32 mm x 0.52 μm film thickness Ultra-1 (Crosslinked Methyl Silicone Gum) Capillary column
Temperature Program	A. 60°C (1 min) to 260°C (25°C/min) B. 260°C (6 min) to 280°C (5 min) (25 °C/min)
Splitless time	1.0 min
Split Ratio	25 : 1
Flow Rate of: Carrier Gas(He)	1.60 mL/min
H_2	45.00 mL/min
Air	380.00 mL/min
N_2	60.00 mL/min
Detector	Flame Ionization Detector
Detector Temperature	300°C
Inlet Temperature	280°C

Table 3.2 The gas chromatographic condition for the study by GC-ECD

GC Parameter	GC Condition
Analytical Column	25 m x 0.32 mm x 0.52 μm film thickness Ultra-1 (Crosslinked Methyl Silicone Gum) Capillary column
Temperature Program	A. 60°C (1 min) to 260°C (25°C/min) B. 260°C (6 min) to 280°C (5 min) (25°C/min)
Splitless time	1.0 min
Split Ratio	25 : 1
Flow Rate of: Carrier Gas(He)	1.60 mL/min
Auxillary Gas (N_2)	60.00 mL/min
Detector	Electron Capture Detector
Detector Temperature	300°C
Inlet Temperature	280°C

5. The Study of Linearity of Standard PEs by GC-ECD

The linearity of phthalate esters were determined by GC-ECD condition in Table 3.2. The concentrations of each phthalate ester were in the range of 0.1 ppm to 100 ppm.

The procedure for the study of linearity of standard PEs by GC-ECD as a detector.

5.1 The concentration of standard solutions PEs 0.10, 0.20, 0.40, 0.60, 0.80, 1.00, 10.00, 20.00, 40.00, 60.00, 80.00 and 100.00 ppm were prepared from the 200.00 ppm stock mixture standard PEs in ethyl acetate.

5.2 1 μL of each concentration was injected in GC-ECD.

5.3 Plot the concentration against peak area.

6. The Procedure of Activated C_{18} SPEM or/and Disk Conditioning

An inevitable condition for effective retention was perfect mutual contact between the solid and the liquid phase. The C_{18} SPEM must be activated before use. Activation of chemically bonded silica causes opening the hydrocarbon chains of the stationary phase, thus increasing its surface area. The C_{18} SPEM was activated by the addition of 10.00 mL of methanol which was allowed to stand for 3 min prior to being drawn off, were not allowing the disk to run dry. The disk was then flushed with 10.00 mL of double distilled water. The solution was passed through the disk with vacuum at 5 mmHg pressure (flow rate : 4-5 mL/min). The materials used in this SPEM technique were shown in Appendix B.

7. The Procedure of Clean Up Step

This study used the modified clean up step of EPA method 506 (74). It was a chromatographic separation method and could be described as follows:

7.1 Florisil 2.0 g was placed (see 2.3) into a minicolumn with the 1.0 mm glassfiber prefilter at the end and added 1.0 g of anhydrous sodium sulphate on the top.

7.2 Pre-elute the minicolumn with 6.00 mL of hexane. Discard the eluate and just prior to exposure of the sodium sulphate layer to the air,

quantitatively transfer the sample extract (0.5-1.0 concentrated eluant) onto the minicolumn, using an additional 2.00 mL of hexane to complete the transfer. Just prior to exposure of the sodium sulphate layer to the air.

7.3 Elute the column with 20% ethyl acetate in hexane (v/v) into a 25 mL beaker. Elute the column at a rate of about 1 mL/min. Evaporate the eluant under nitrogen stream.

8. The Procedure for Preparation of Spiked Standard Milk

Three volumes, 25.00, 50.00, 100.00 g of standard milk were prepared in this study and could be described as follows:

8.1 For method 1-5, 5.00 μL (low concentration) and 0.50 mL (high concentration) of 250.00 ppm mixture standard solution in methanol were pipetted into 25.00 mL volumetric flask and then filling it to the mark with standard milk.

8.2 For method 6, 10.00 μL (low concentration) and 1.00 mL (high concentration) of 250.00 ppm mixture standard solution in methanol were pipetted into 50.00 mL volumetric flask and then filling it to the mark with standard milk.

8.3 For method 7-8, 20.00 μL (low concentration) and 2.00 mL (high concentration) of 250.00 ppm mixture standard solution in methanol were pipetted into 100.00 mL volumetric flask and then filling it to the mark with standard milk.

Blank standard milk of each method was prepared as same as spiked standard milk but was not added the mixture standard PEs.

9. The Study of Various Methods on the Time Requirement, Percent Recovery, Precision, Method Detection Limit for Preparation of Milk Sample by SPEM

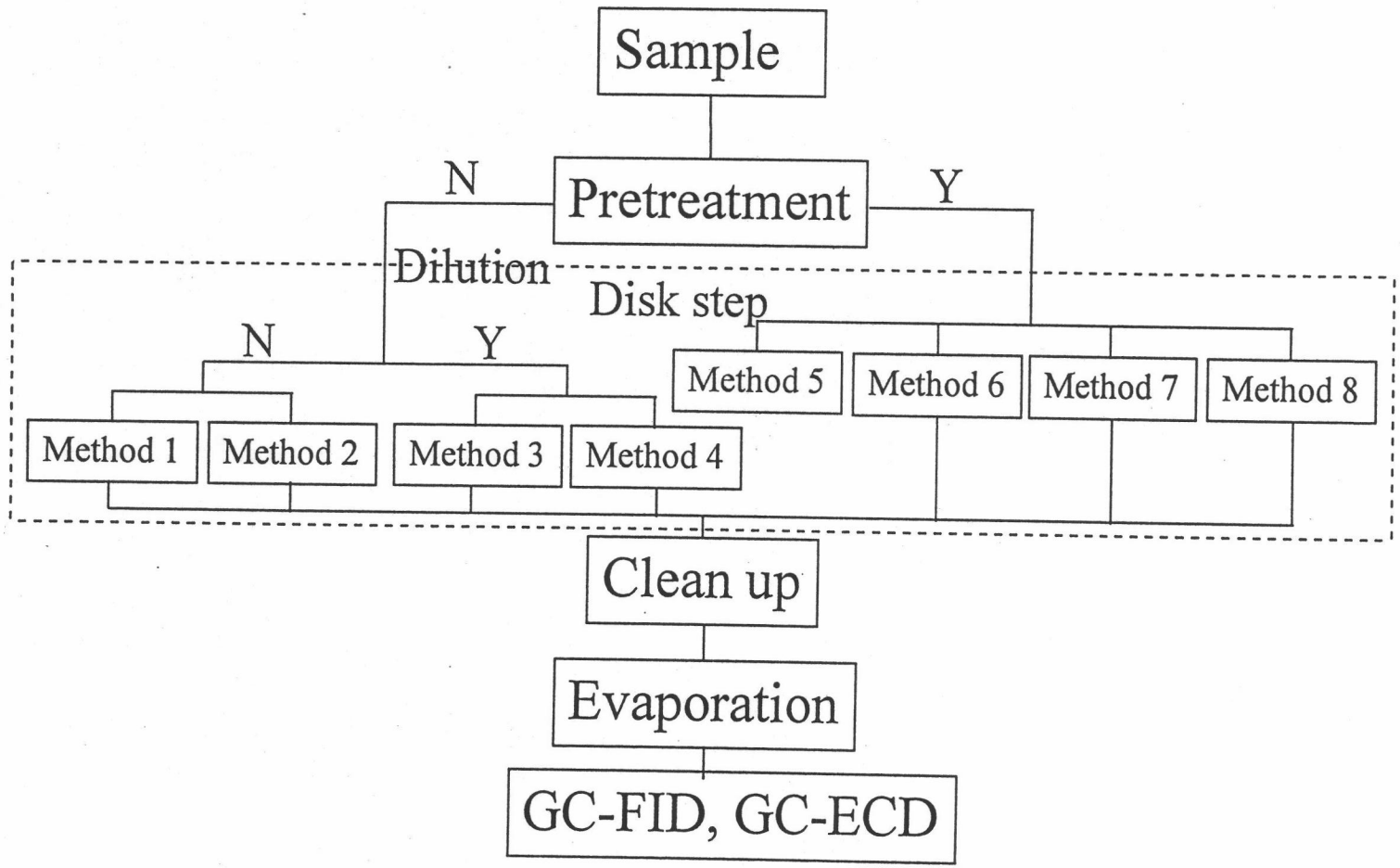
The time requirement was the minimum total time that was spended in the procedure of preparation in each method.

Percent Recovery was the key word to explain how many percent of analytes in sample which the preparation method could be observed, when was defined that the amount in fact was equal to 100 percent.

Precision describes the agreement between two or more measurements that have been made in exactly the sameway there were several ways to express precision. The standard deviation was a statistical term used as a measurement of precision.

The various methods in this study combined the SPEM and the preparation method for milk to new preparative method.

Procedures


Sample
Pretreatment
N
Y
Dilution
Disk step
N
Y
Method 1
Method 2
Method 3
Method 4
Method 5
Method 6
Method 7
Method 8
Clean up
Evaporation
GC-FID, GC-ECD

9.1 Method 1: Direct Sample Throughput Method

The Procedure for the study in method 1 of two concentration 0.05 and 5.00 mg/kg on the percent recovery, precision method detection limit could be described as follows:

For the study of time requirement, percent recovery, precision, three of low concentration, three of high concentration of spiked standard milk and blank standard milk in 8.1 were used. Collect the spending time in method 1-8.

9.1.1 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. The disk was washed with 10.00 mL ethyl acetate by adding ethyl acetate to the disk, passing about 3 min, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.1.2 The disk was conditioned with 10.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about a minute, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.1.3 The disk was rinsed with double distilled water by adding the water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.1.4 The milk sample was added to the reservior and turned on the vacuum to begin the filtration. Full aspirate vacuum might be used.The flow rate was adjusted in range of 4-5 mL/min and allowed it to dry 3 min. The disk

was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about half of the hexane through the disk and allowing it to soak for a minute, then passing most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.1.5 The PEs from disk were eluted: remove the filtration top from the vacuum (suctional) flask, but did not disassemble the reservoir and fritted base. Empty the milk from the flask and insert 25x200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

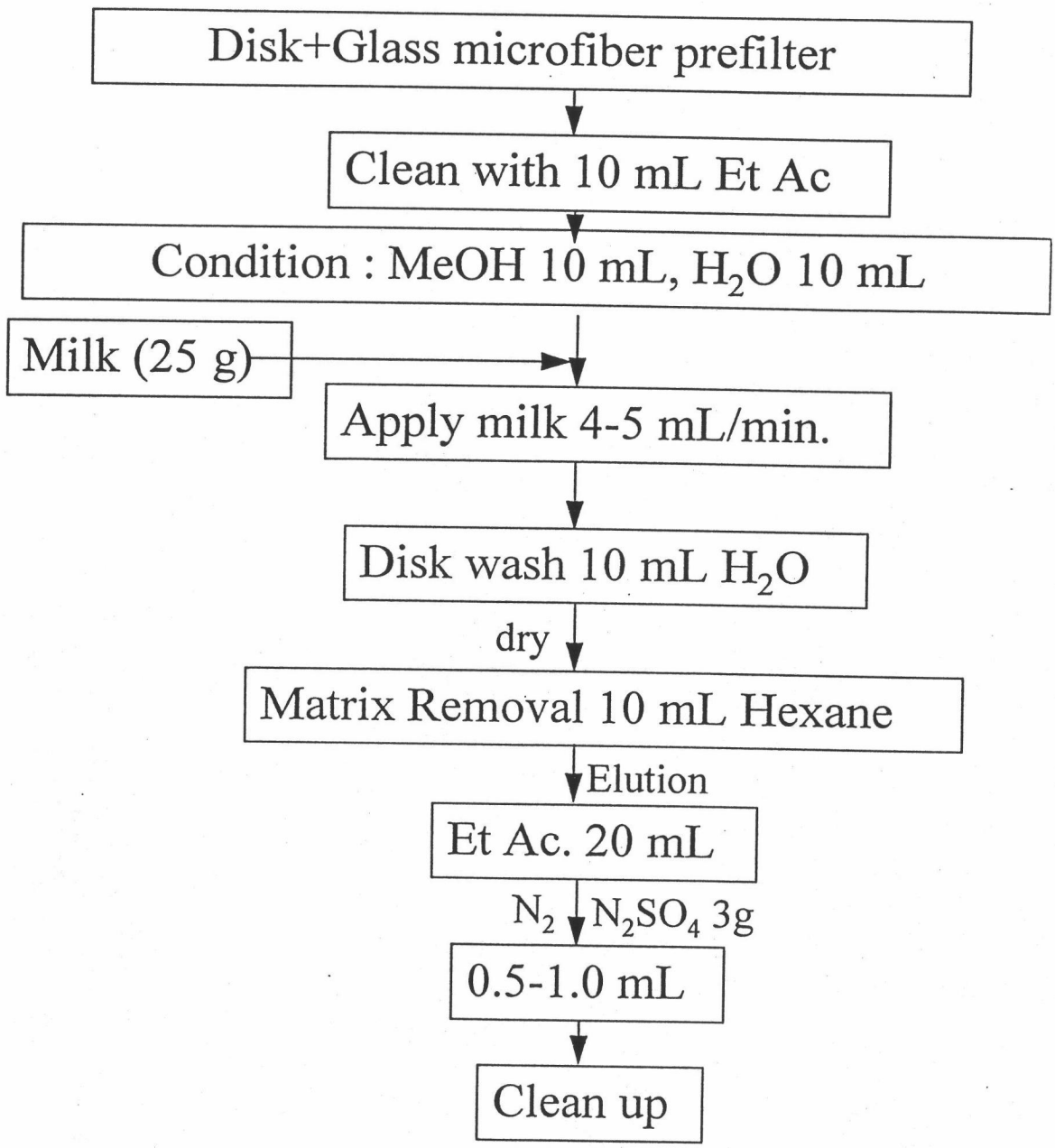
10.00 mL of ethyl acetate were added to the disk. Ethyl acetate was allowed to settle and passed about half of ethyl acetate through the disk, it was allowed to soak for a minute. The second 10.00 mL of ethyl acetate was added to the disk and passed the remaining ethyl acetate through the disk.

9.1.6 The eluant was transfered from 9.1.5 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.1.7 The eluant in 9.1.6 was concentrated and cleaned up by the method in item 7: the procedure of clean up step, then transferred into 5.00 mL volumetric flask and made volume until to the mark and both concentration were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.1.8 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 1


Disk+Glass microfiber prefilter
Clean with 10 mL Et Ac
Condition : MeOH 10 mL, H_2O 10 mL
Milk (25 g)
Apply milk 4-5 mL/min.
Disk wash 10 mL H_2O
dry
Matrix Removal 10 mL Hexane
Elution
Et Ac. 20 mL
N_2
N_2SO_4 3g
0.5-1.0 mL
Clean up

9.2 Method 2: Dilute Sample Method

Like the method 1 (9.1), three low concentration of spiked standard and three high concentration of spiked standard which were prepared in step 8.1 were used in this study, and could be described as follows:

9.2.1 Every 25.00 g spiked standard milk was diluted to 10.00 mL with double distilled water and used it in the following steps.

9.2.2 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. The disk was washed with 10.00 mL ethyl acetate by adding ethyl acetate to the disk, passing about three minutes, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.2.3 The disk was conditioned with 10.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about a minute, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.2.4 The disk was rinsed with double distilled water by adding the water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.2.5 The milk sample was added to the reservior and turned on the vacuum to begin the filtration. Full aspirate vacuum might be used.The flow rate was adjusted in range of 4-5 mL/min and allowed it to dry 3 min. The disk was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about half of hexane through the disk and allowing it to soak for a minute, then

passing most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.2.6 The PEs from disk were eluted: remove the filtration top from the vacuum (suctional) flask, but did not disassemble the reservoir and fritted base. Empty the milk from the flask and insert 25x200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

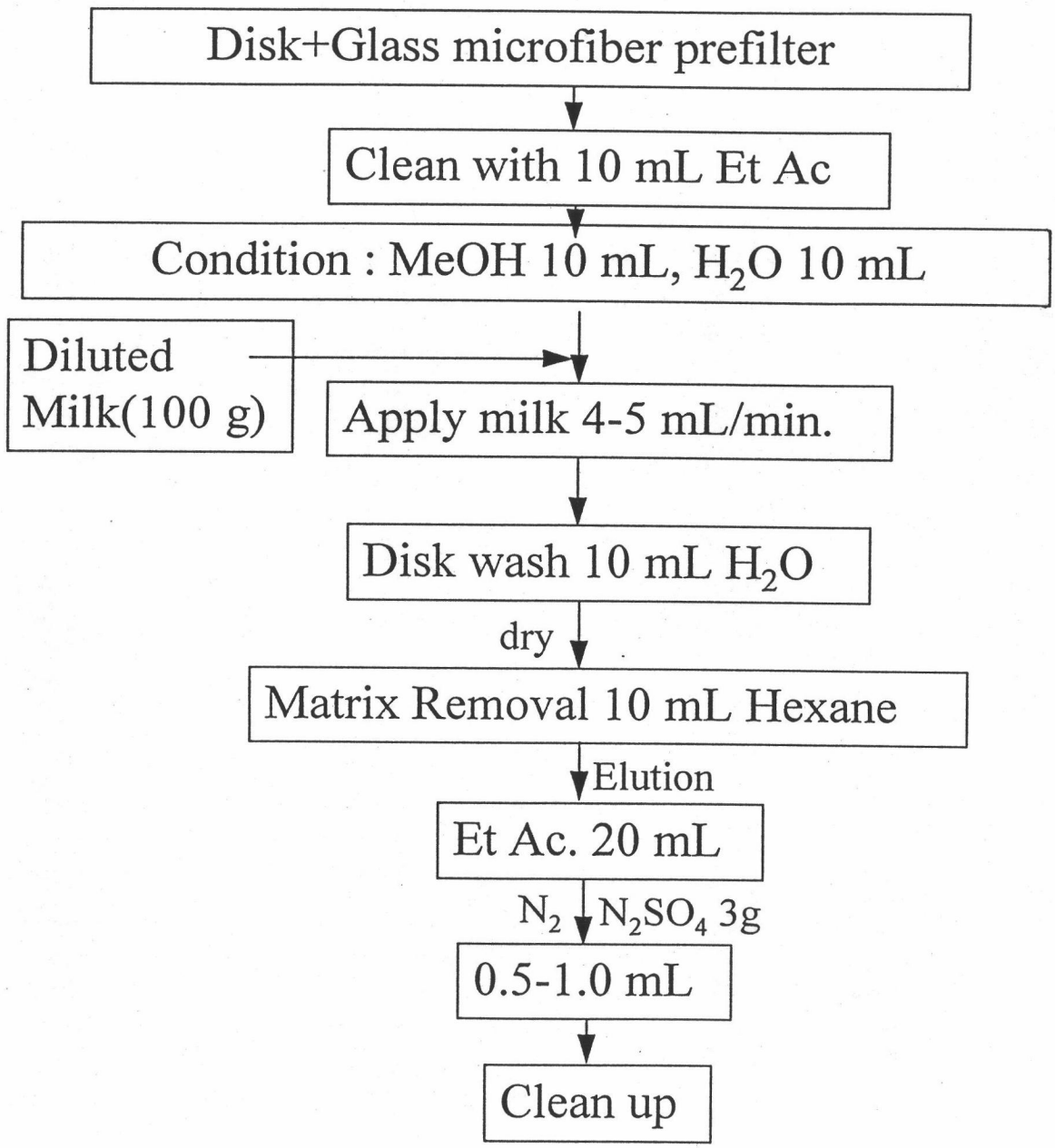
10.00 mL of ethyl acetate were added to the disk. Ethyl acetate was allowed to settle and passed about half of ethyl acetate through the disk, it was allowed to soak for a minute. The second 10.00 mL of ethyl acetate was added to the disk and pass the remaining ethyl acetate through the disk.

9.2.7 The eluant was transferred from 9.2.6 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.2.8 The eluant in 9.2.7 was concentrated and was cleaned up by the method in item 7: the procedure of clean up step, then transferred it into 5.00 mL volumetric flask and made volume until to the mark and both concentrations were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.2.9 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 2


Disk+Glass microfiber prefilter
Clean with 10 mL Et Ac
Condition : MeOH 10 mL, H_2O 10 mL
Diluted Milk(100 g)
Apply milk 4-5 mL/min.
Disk wash 10 mL H_2O
dry
Matrix Removal 10 mL Hexane
Elution
Et Ac. 20 mL
N_2
N_2SO_4 3g
0.5-1.0 mL
Clean up

9.3 Method 3: Filter Aid with SPEM

Three low concentration, three high concentration spiked in standard and blank standard milk which were prepared in step 8.1 were used in the next-step as follows:

9.3.1 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. Gradually pour 12.0 g of Filter Aid on to the disk. The three layers (Filter Aid, glass microfiber fiter and disk) were washed with 20.00 mL of ethyl acetate by adding ethyl acetate to the three layers, passing about half of ethyl acetate through the disk and allowing it to soak the disk for about a minute, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.3.2 The disk was conditioned with 20.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about 3 min, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.3.3 Rinse the disk with double distillated water by adding the water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.3.4 The milk sample was added to the reservior and turned on the vacuum to begin the filtration. Full aspirate vacuum might be used.The flow rate was adjusted in range of 4-5 mL/min and allowed it to dry 3 min. The disk was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about

half of hexane through the disk and allowing it to soak for 3 min, then passing most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.3.5 The PEs from disk were eluted : remove the filtration top from the vacuum (suctional) flask, but do not disassemble the reservior and fritted base. Empty the milk from the flask and insert 25 x 200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

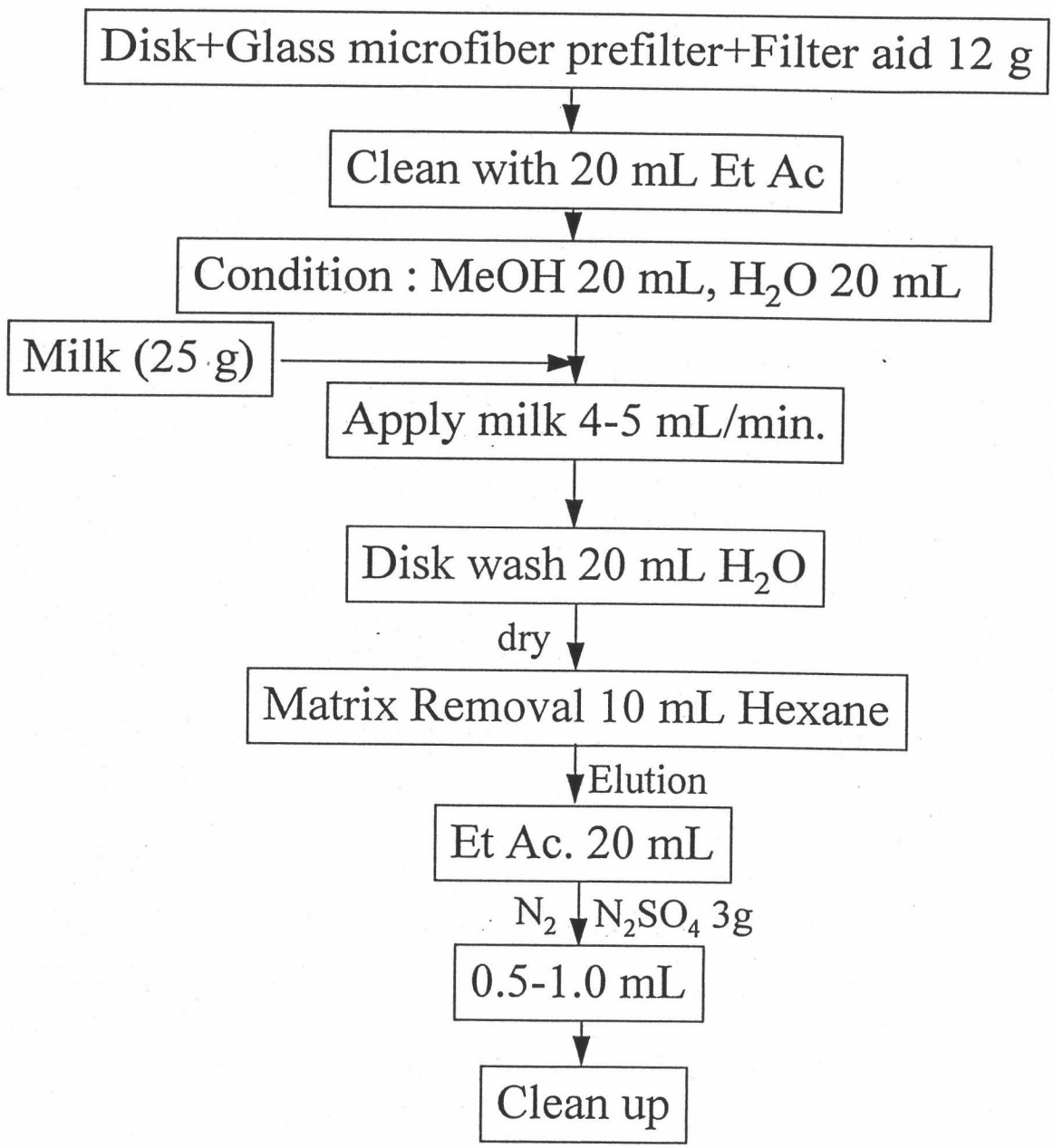
10.00 mL of ethyl acetate were added to the disk. Ethyl acetate was allowed to settle and passed about half of ethyl acetate through the disk, it was allowed to soak for a minute. The second 10.00 mL of ethyl acetate was added to the disk and pass the remaining ethyl acetate through the disk.

9.3.6 The eluant was transferred from 9.3.5 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.3.7 The eluant in 9.3.6 was concentrated and cleaned up by the method in item 7: the procedure of clean up method, then transferred it into 5.00 mL volumetric flask and made volume until to the mark and both concentrations were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.3.8 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 3


Disk+Glass microfiber prefilter+Filter aid 12 g
Clean with 20 mL Et Ac
Condition : MeOH 20 mL, H_2O 20 mL
Milk (25 g)
Apply milk 4-5 mL/min.
Disk wash 20 mL H_2O
dry
Matrix Removal 10 mL Hexane
Elution
Et Ac. 20 mL
N_2
N_2SO_4 3g
0.5-1.0 mL
Clean up

9.4 Method 4: Dilute Sample with Filter Aid

Similar to the method 9.3, three low concentration of spiked standard and three high concentration of spiked standard which were prepared in step 8.1 were used in this study, and could be described as follows:

9.4.1 Dilute 25.00 g of every spiked standard milk to 100.0 mL with double distilled water and used it in the following steps:

9.4.2 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. Gradually pour 12.0 g of Filter Aid on to the disk. The three layers (Filter Aid, glass microfiber fiter and disk) were washed with 20.00 mL of ethyl acetate by adding ethyl acetate to the three layers, passing about half of ethyl acrtate through the disk and allowing it to soak the disk for about a minute, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.4.3 The disk was conditioned with 20.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about 3 min, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.4.4 Rinse the disk with double distilled water by adding the water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.4.5 The milk sample was added to the reservior and turned on the vacuum to begin the filtration. Full aspirate vacuum might be used.The flow

rate was adjusted in range of 4-5 mL/min and allowed it to dry 3 min. The disk was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about half of hexane through the disk and allowing it to soak for 3 min, then passing most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.4.6. The PEs from disk were eluted: remove the filtration top from the vacuum (suctional) flask, but did not disassemble the reservior and fritted base. Empty the milk from the flask and insert 25 x 200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

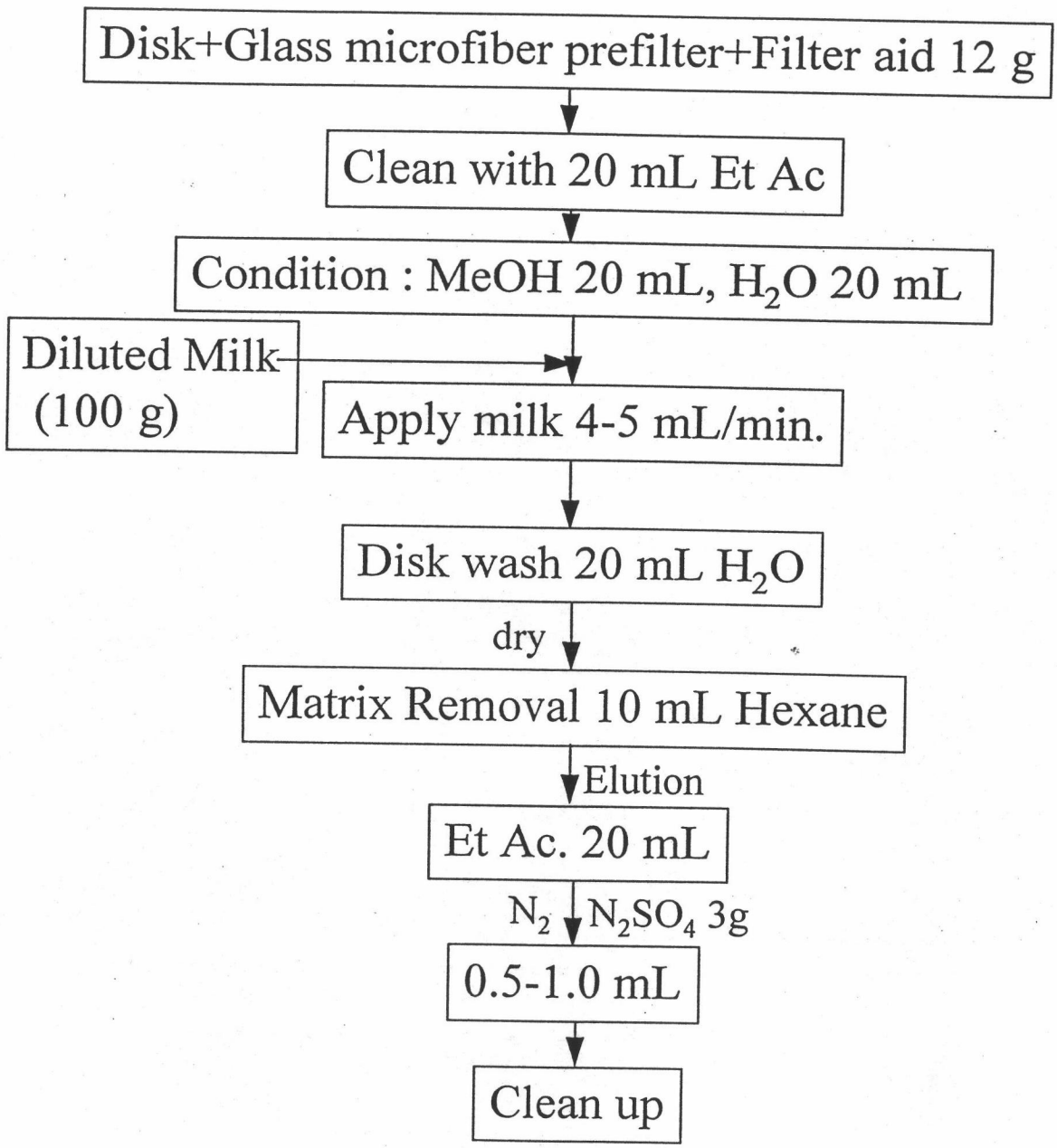
10.00 mL of ethyl acetate were added to the disk. Ethyl acetate was allowed to settle and passed about half of ethyl acetate through the disk, it was allowed to soak for a minute. The second 10.00 mL of ethyl acetate was added to the disk and pass the remaining ethyl acetate through the disk.

9.4.7 The eluant was transferred from 9.4.6 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.4.8 The eluant in 9.4.7 was concentrated and cleaned up by the method in item 7; the procedure of clean up method, then transferred it into 5.00 mL volumetric flask and made volume until to the mark and both concentrations were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.4.9 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 4


Disk+Glass microfiber prefilter+Filter aid 12 g
Clean with 20 mL Et Ac
Condition : MeOH 20 mL, H_2O 20 mL
Diluted Milk (100 g)
Apply milk 4-5 mL/min.
Disk wash 20 mL H_2O
dry
Matrix Removal 10 mL Hexane
Elution
Et Ac. 20 mL
N_2
N_2SO_4 3g
0.5-1.0 mL
Clean up

9.5 Method 5: Solvent Extraction with SPEM

Three low concentration and three high concentration level spiked in standard milk in 8.1 were prepared in this method.

9.5.1 All 25.00 g standard milks were mixed well with 100.0 mL of ternary solvent (ethyl acetate:ethanol:acetone, 2:4:4) and vortexed for a minute. All sample were then placed in ultrasonic bath for 20 min.

9.5.2 The sample were centrifuged at 2000 rpm for 15 min at 20°C and the total supernatant were transferred into 500 mL conical flasks.

9.5.3 An aliquot of 250 mL of double distilled water was added to the supernatants.

9.5.4 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. The disk was washed with 10.00 mL ethyl acetate by adding ethyl acetate to the disk, passing about 3 min, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.5.5 The disk was conditioned with 10.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about a minute, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.5.6 The disk was rinsed with double distilled water by adding the water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.5.7 The diluted supernatant was passed through the disk at a flow rate 4-5 mL/min and allowed it to dry 3 min. The disk was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about half of hexane through the disk and allowing it to soak for a minute, the passing most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.5.8 The PEs from disk were eluted: remove the filtration top from the vacuum (suctional) flask, but did not disassemble the reservoir and fritted base. Empty the milk from the flask and insert 25x200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

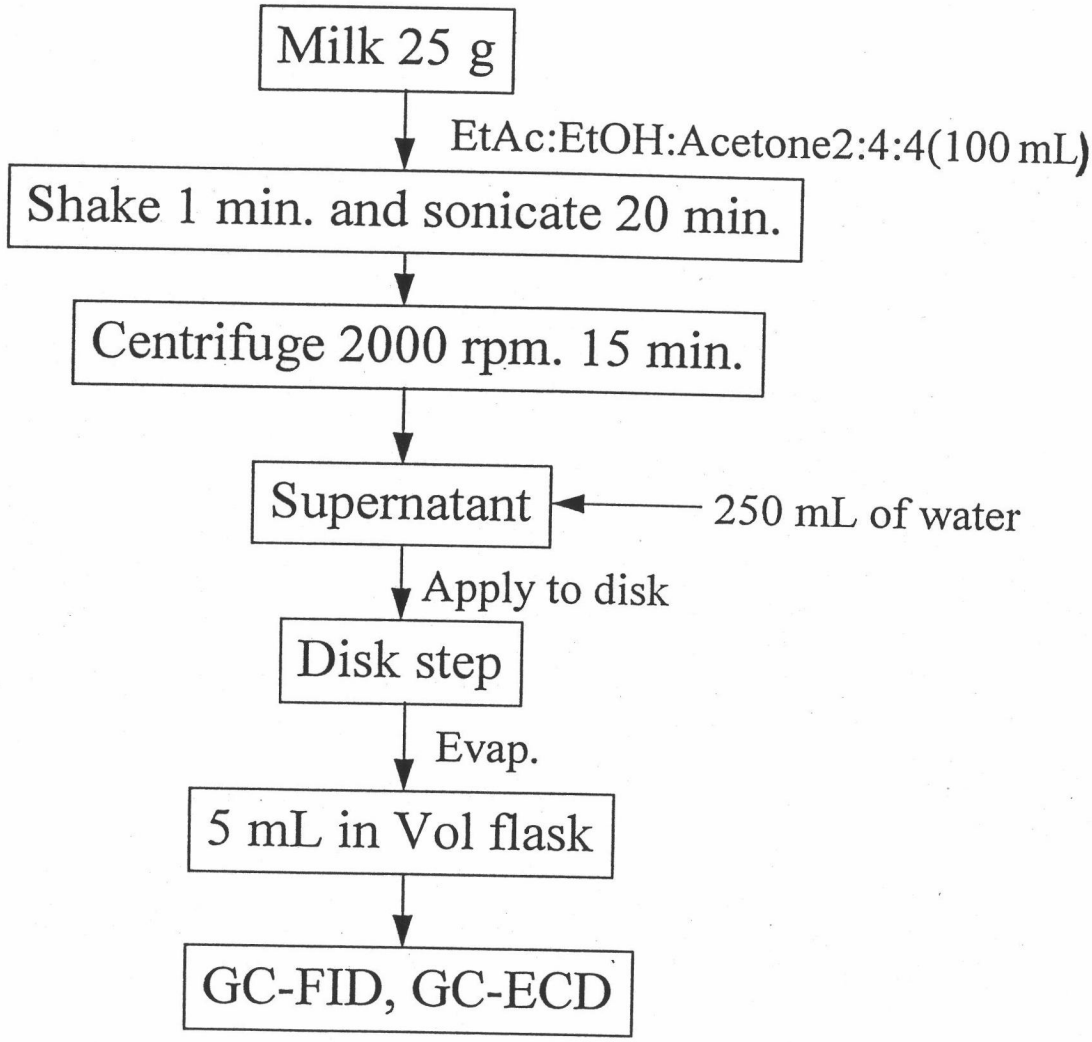
10.00 mL of ethyl acetate were added to the disk. Ethyl acetate was allowed to settle and passed about half of ethyl acetate through the disk, it was allowed to soak for a minute. The second 10.00 mL of ethyl acetate was added to the disk and passed the remaining ethyl acetate through the disk.

9.5.9 The eluant was transferred from 9.5.8 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.5.10 The eluant in 9.5.9 was concentrated and cleaned up by the method in item 7: the procedure of clean up method, then transferred it into 5.00 mL volumetric flask and made volume until to the mark and both concentrations were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.5.11 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 5


Milk 25 g
EtAc:EtOH:Acetone2:4:4(100 mL)
Shake 1 min. and sonicate 20 min.
Centrifuge 2000 rpm. 15 min.
Supernatant
250 mL of water
Apply to disk
Disk step
Evap.
5 mL in Vol flask
GC-FID, GC-ECD

9.6 Method 6: Acidic Adjustment with Sample

The spiked standard milks were adjusted to acidic condition with pH 2.10 before they were applied to SPEM.

9.6.1 Three low concentration and three high concentration of spiked standard milks were prepared in 8.2 were used in the following steps.

9.6.2 Each 50.00 g of standard milk was diluted to 100 mL and adjusted to pH 2.10 by 6 N hydrochloric acid and 6 N sodium hydroxide drop wise.

9.6.3 The adjusted milk was then centrifuged at 3500 rpm for 10 min at 5°C.

9.6.4 The supernatant was passes through the disk in following steps.

9.6.5 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. The disk was washed with 10.00 mL ethyl acetate by adding ethyl acetate to the disk, passing about 3 min, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.6.6 The disk was conditioned with 10.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about a minute, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.6.7 The disk was rinsed with double distilled water by adding water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.6.8 The water sample was added to the reservior and turned on the vacuum to begin the filtration. Full aspirate vacuum might be used.The flow rate was adjusted in range of 4-5 mL/min and allowed it to dry 3 min. The disk was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about half of hexane through the disk and allowing it to soak for a minute, the passing most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.6.9 The PEs from disk were eluted: remove the filtration top from the vacuum (suctional) flask, but did not disassemble the reservoir and fritted base. Empty the milk from the flask and insert 25x200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

10.00 mL of ethanol and 10.00 mL of ethyl acetate were added to the precipitate of centrifigation part and then it was sonicated for 10 min. Centrifugation at 2000 rpm for a minute was used to settle the extraction and then it was rinsed with 10.00 mL of ethyl acetate. The total extraction was transferred on to the disk, rinsed it again with 10.00 mL of ehyl acetate. Ethyl acetate was allowed to extract and passed about half of ethyl acetate through the disk, and allowed it to soak for a minute.

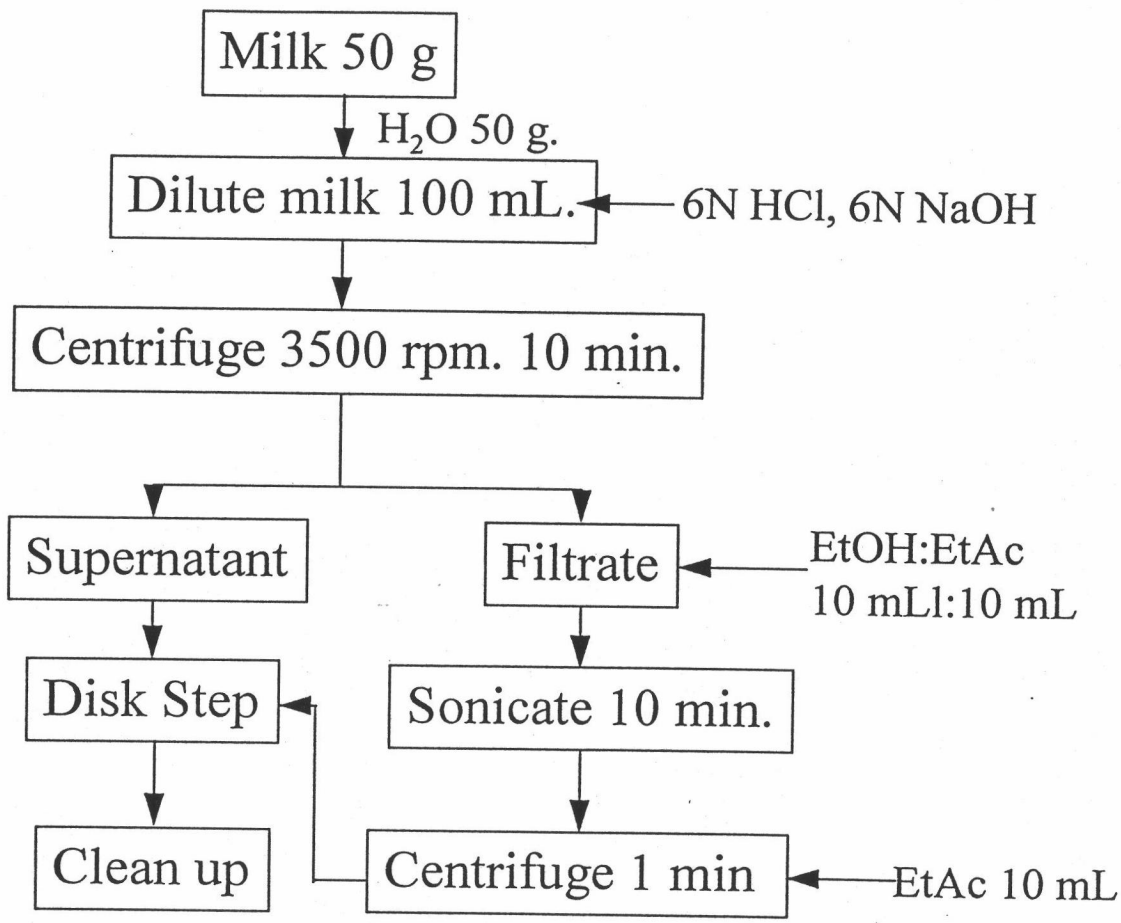
9.6.10 The eluant was transferred from 9.6.9 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.6.11 The eluant in 9.6.10 was concentrated and cleaned up by the method in item 7: the procedure of clean up method, then transferred it into 5.00 mL volumetric flask and made volume until to the mark and both

concentrations were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.6.12 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 6


Milk 50 g
H_2O 50 g.
Dilute milk 100 mL.
6N HCl, 6N NaOH
Centrifuge 3500 rpm. 10 min.
Supernatant
Filtrate
EtOH:EtAc
10 mLl:10 mL
Disk Step
Sonicate 10 min.
Clean up
Centrifuge 1 min
EtAc 10 mL

9.7 Method 7: Centrifugation Preparation

Three low concentration and three high concentration level spiked standard milk in 8.3 were prepared.

9.7.1 All 100.0 g of milk samples were transferred into rotor tube of ultracentrifugation and then were centrifuged with 18,000 rpm for 30 min at 4°C.

9.7.2 Separate the liquid phase and solid phase and only the supernatant was passed through the disk in following steps

9.7.3 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. The disk was washed with 10.00 mL ethyl acetate by adding ethyl acetate to the disk, passing about 3 min, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.7.4 The disk was conditioned with 10.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about a minute, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.7.5 The disk was rinsed with double distilled water by adding the water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.7.6 The water sample was added to the reservior and turned on the vacuum to begin the filtration. Full aspirator vacuum might be used. The flow rate was adjusted in range of 4-5 mL/min and allowed it to dry 3 min. The

disk was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about half of hexane through the disk and allowing it to soak for a minute, the passing most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.7.7 The PEs from disk were eluted: remove the filtration top from the vacuum (suctional) flask, but did not disassemble the reservoir and fritted base. Empty the milk from the flask and insert 25x200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

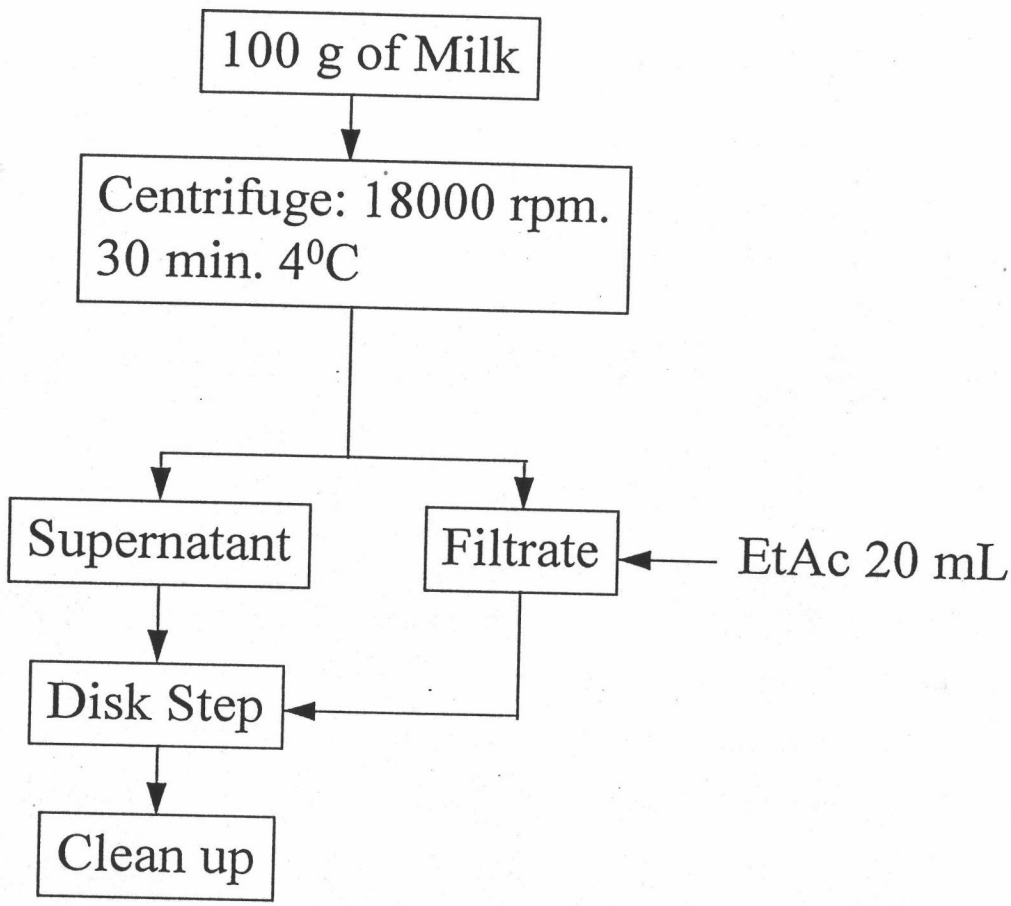
20.00 mL of ethyl acetate was added to the precipitate of centrifugation part and stirred it for a minute and the ethyl acetate extraction was transferred onto disk, rinsed it again with 10.00 mL of ethyl acetate. Allow ethyl acetate extraction to settle and pull about half of ethyl acetate through the disk, and allow it to soak for a minute.

9.7.8 The eluant was transfered from 9.7.7 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.7.9 The eluant in 9..7.8 was concentrated and cleaned up by the method in item 7: the procedure of clean up method, then transferred it into 5.00 mL volumetric flask and made volume until to the mark and both concentrations were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.7.10 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 7(Centrifuge)



9.8 Method 8: Milk Precipitation

The precipitation of protein was the popular method for sample preparation in milk. Many precipitation methods were used to prepare sample. In this study used the Schuffenburg's method and could be described as followed:

9.8.1 Three low concentration and three high concentration of spiked standard milk which were prepared in 8.3 were used in the following steps.

9.8.2 Each 100.0 g of standard milk was added 100.0 mL of water and 10.00 mL of 10% (w/w) acetic acid. The solution was mixed well for 10 min, and then 10.00 mL of 1M sodium acetate in water were added and mixed for 15 min. The mass was adjusted to 250 g with water. The precipitate was removed by centrifugation at 2000 g for 20 min at 5°C.

9.8.3 The supernatant was passed through the disk in following step.

9.8.4 The disk was inserted into the 47 mm filter apparatus and the 47 mm glass microfiber filter was placed cover on the disk. The disk was washed with 10.00 mL ethyl acetate by adding ethyl acetate to the disk, passing about 3 min, then passing the remaining ethyl acetate through the disk. With the vacuum on, pull air through the disk for a minute.

9.8.5 The disk was conditioned with 10.00 mL methanol by adding the methanol to the disk, passing about half of methanol through the disk and allowing it to soak for about a minute, then passing most of the remaining methanol through the disk. The layer of methanol must be left on the surface of the disk, which should not be allowed to go dry from this point until the end of the sample extraction. This was a critical step for a uniform flow and good recovery.

9.8.6 The disk was rinsed with double distilled water by adding the water to the disk and passing most through, again leaving the layer on the surface of the disk.

9.8.7 The milk sample was added to the reservior and turned on the vacuum to begin the filtration. Full aspirate vacuum might be used.The flow rate was adjusted in range of 4-5 mL/min and allowed it to dry 3 min. The disk was rinsed with 10.00 mL hexane by adding hexane to the disk, passing about half of hexane through the disk and allowing it to soak for a minute, the pulling most of the remaining hexane through and the disk was dried completely under vacuum for 5-10 min.

9.8.8 The PEs from disk were eluted: remove the filtration top from the vacuum (suctional) flask, but did not disassemble the reservoir and fritted base. Empty the milk from the flask and insert 25x200 mm test tube to contain the eluant. The only constraint on the sample tube was that it fit around the drip tip of the fritted base. Reassemble the apparatus.

10.00 mL of ethyl acetate were added to the disk. Ethyl acetate was allowed to settle and passed about half of ethyl acetate through the disk, it was allowed to soak for a minute. The second 10.00 mL of ethyl acetate was added to the disk and passed the remaining ethyl acetate through the disk.

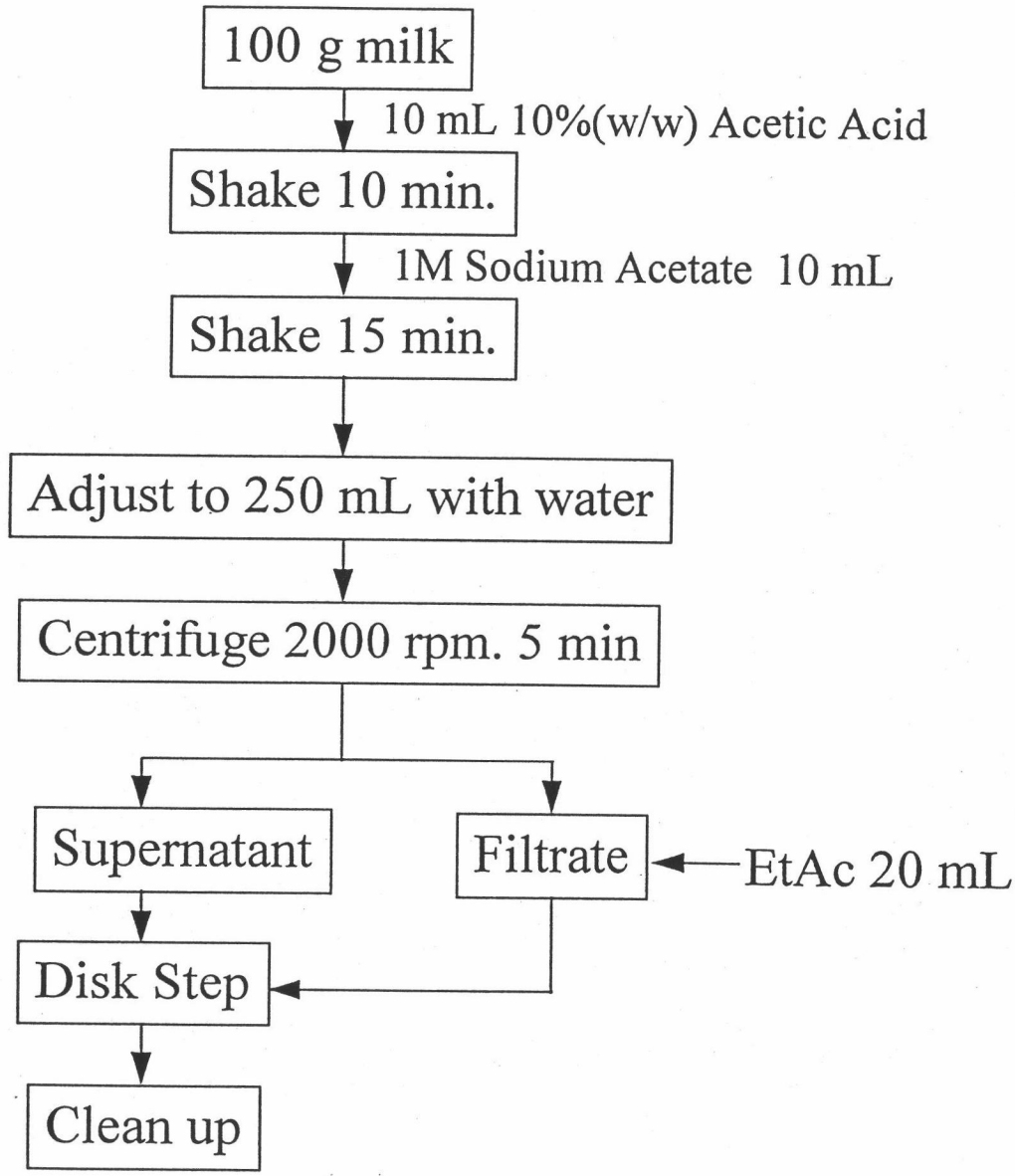
9.8.9 The eluant was transferred from 9.8.8 to 25 mL beaker by pass it through filter paper which contained 3 g of anhydrous sodium sulphate. Concentrate the eluant to 0.50-1.00 mL under nitrogen stream.

9.8.10 The eluant in 9.8.9 was concentrated and cleaned up by the method in item 7: the procedure of clean up method, then transferred it into 5.00 mL volumetric flask and made volume until to the mark and both concentrations were analyzed by GC-FID and only low concentration was analyzed by GC-ECD.

9.8.11 The concentration of each PE was calculated by using the external standardization method. The concentration of standard calibration

curve were 0.10, 0.20, 0.40, 0.60, 0.80, and 1.00 mg/kg for 0.05 mg/kg spiked standard milk and were 10.00, 20.00, 40.00, 60.00, 80.00, and 100.00 mg/kg for 5 mg/kg spiked standard milk.

Method 8 (precipitate)



10. The Study of Various Methods on The Method Detection Limit.

The method detection limit (MDL) was defined as the minimum concentration of a substance that could be identified, measured, and reported with 99% confidence that the analyte concentration was greater than zero and was determined from replicate analyzes for a sample of a given matrix containing analyte. The procedure for the study of method detection limit could be described as follows:

Three standard milks of each PE in each concentration were prepared following the method in 1-8. The extraction of every method was injected into gas chromatography under GC condition as described in Table 3.1 and Table 3.2.

11. External Standardization Method (75)

The technique of external standardizaton involved the preparation of standards at the same levels of concentration as the knowns in the same matrix as the unknowns. A direct relationship between peak size and composition of one or more components could be established, and the unknowns compared graphically or mathematically to the standards for analysis.

Standards could be prepared with all components of interest in each standard, and the range of composition of the standards should cover the entire range expected in the unknowns.

Two very important items could be learned from the calibration curve. In general, the curves were straight lines, and they passed through the origin. These two requirements were most important of the determination that under conditions of analysis and over the concentration range covered: the column

had not been overloaded; the detector had not been overloaded; the electronics were responding linearly; and there was no apparent component adsorption in the injection port, the column, the detector, and associate plumbing. It was generally possible to obtain calibration curves, where the concentration region of interest was linear and where the plot extrapolated through the origin.

Liquid Standard were quite straight forward, and reasonable analytical technique could ensure reliable standards.

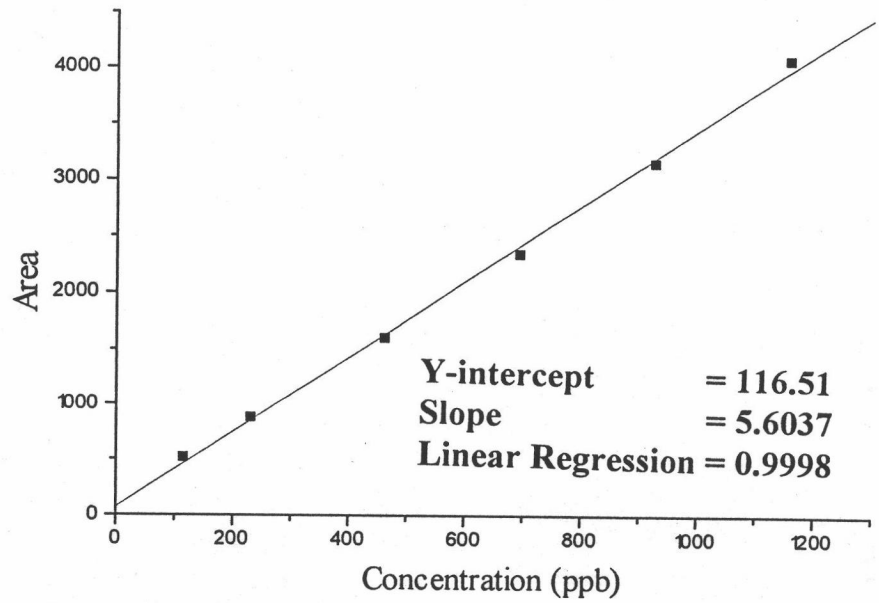


Figure 3.1 The calibration curve of standard DMP (low concentration) by the condition in Table 3.1

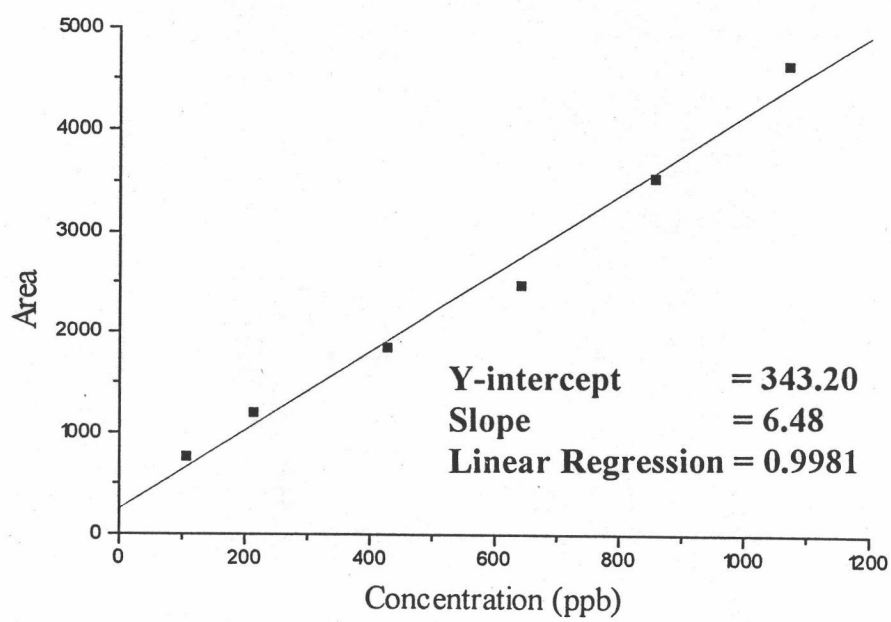


Figure 3.2 The calibration curve of standard DEP (low concentration) by the condition in Table 3.1

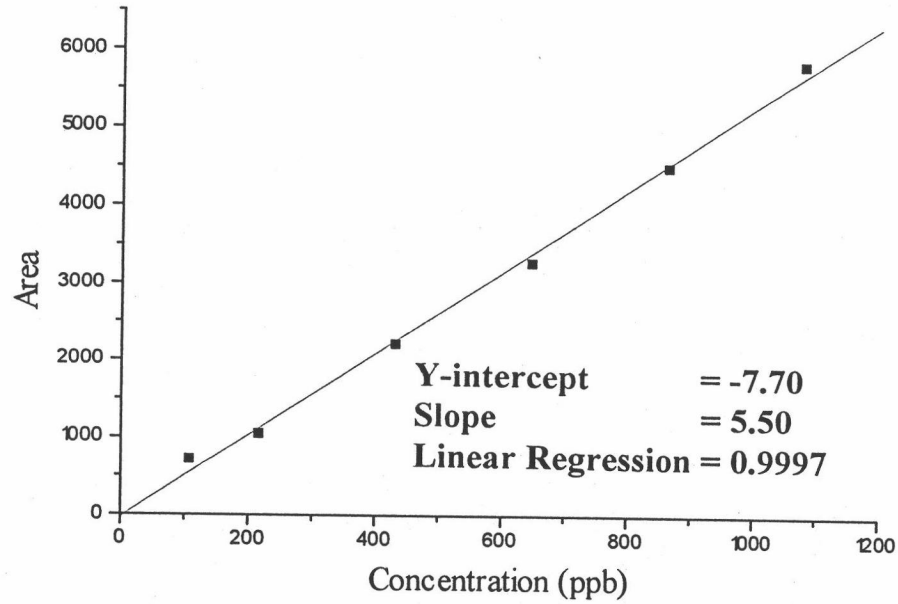


Figure 3.3 The calibration curve of standard DBP (low concentration) by the condition in Table 3.1

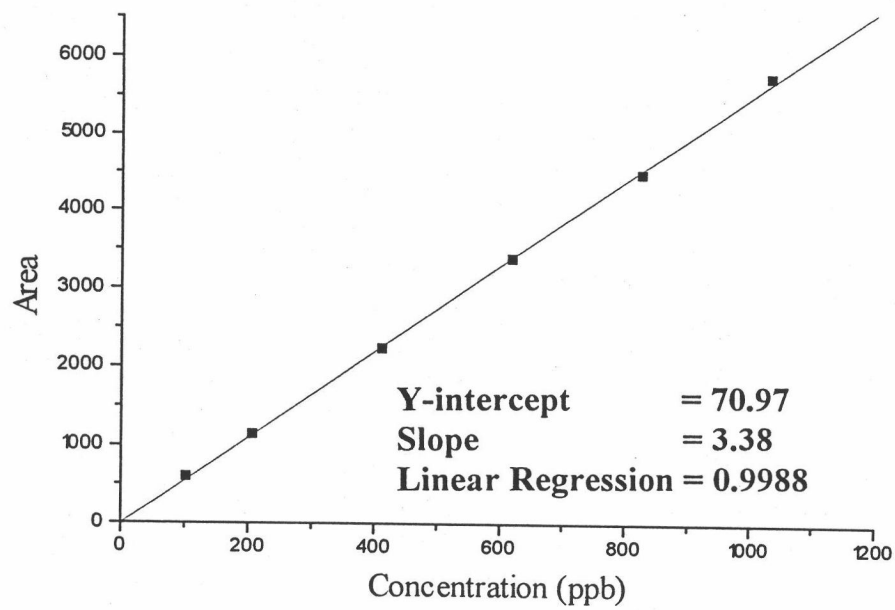


Figure 3.4 The calibration curve of standard BBP (low concentration) by the condition in Table 3.1

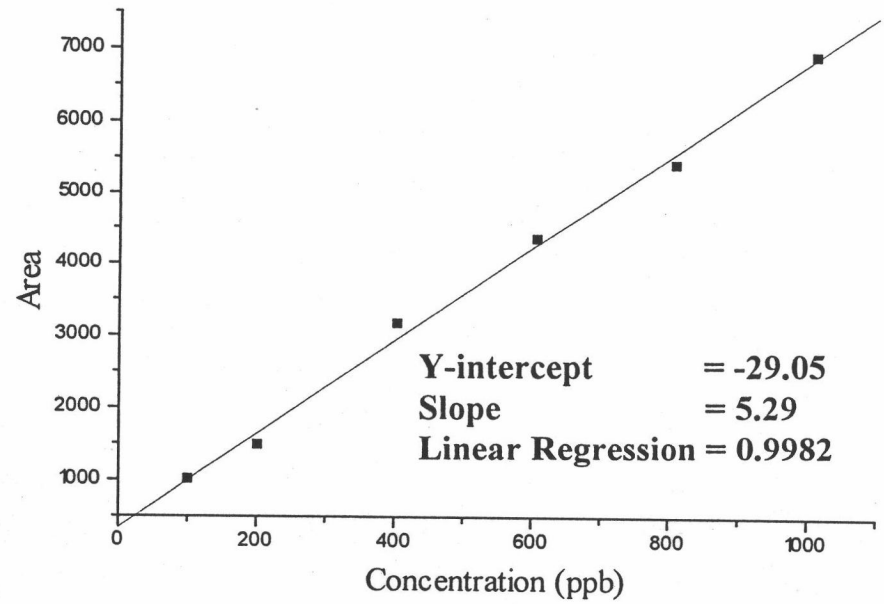


Figure 3.5 The calibration curve of standard DEHP (low concentration) by the condition in Table 3.1

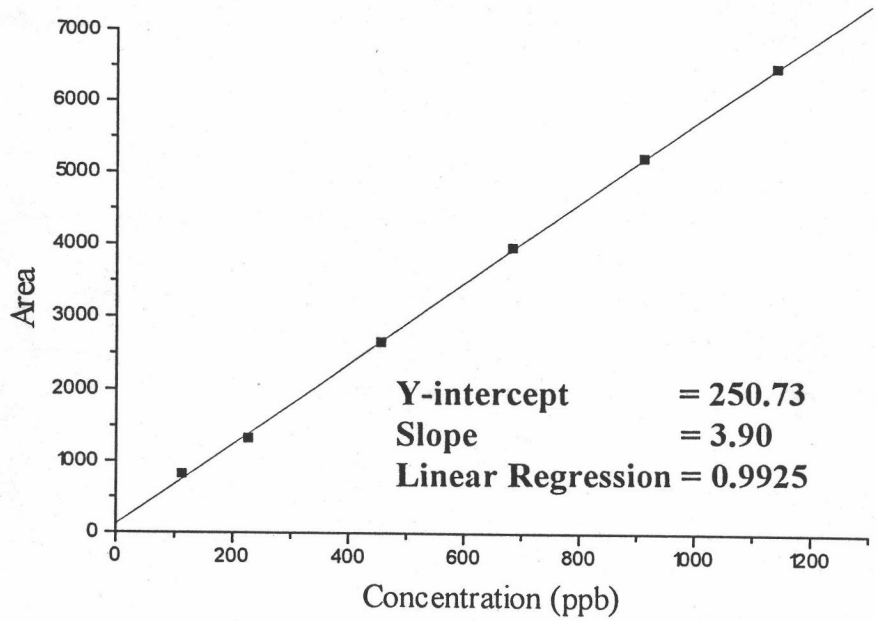


Figure 3.6 The calibration curve of standard DOP (low concentration) by the condition in Table 3.1

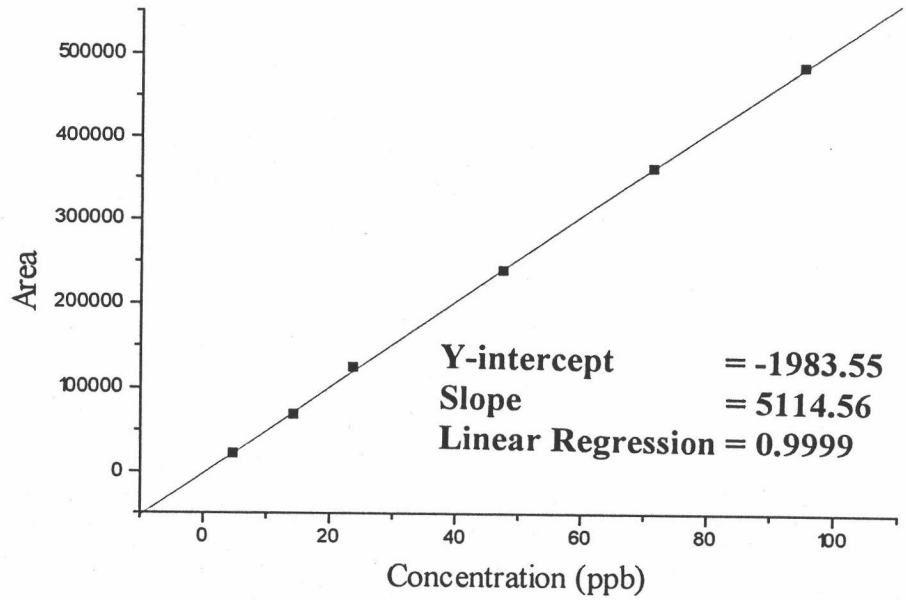


Figure 3.7 The calibration curve of standard DMP (high concentration) by the condition in Table 3.1

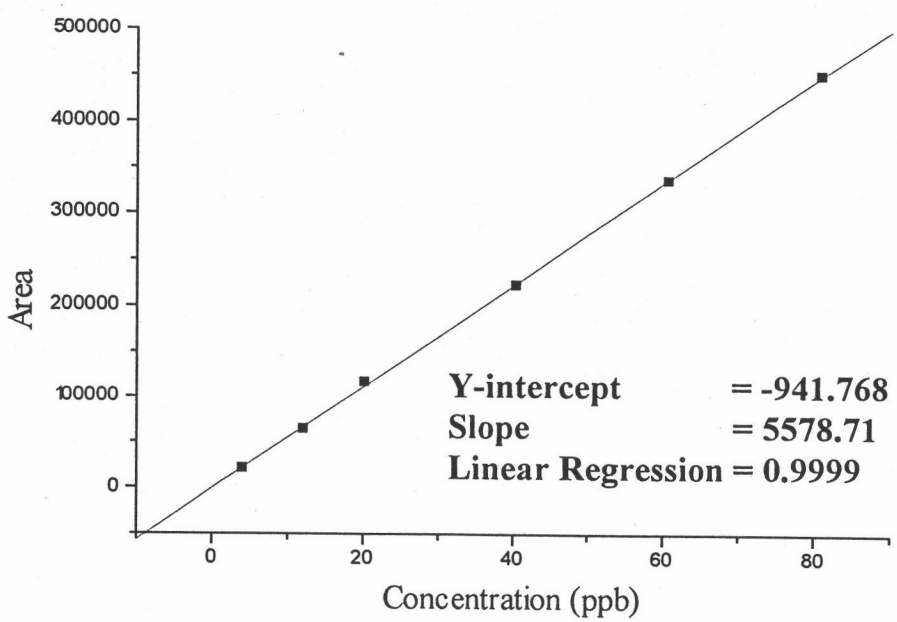


Figure 3.8 The calibration curve of standard DEP (high concentration) by the condition in Table 3.1

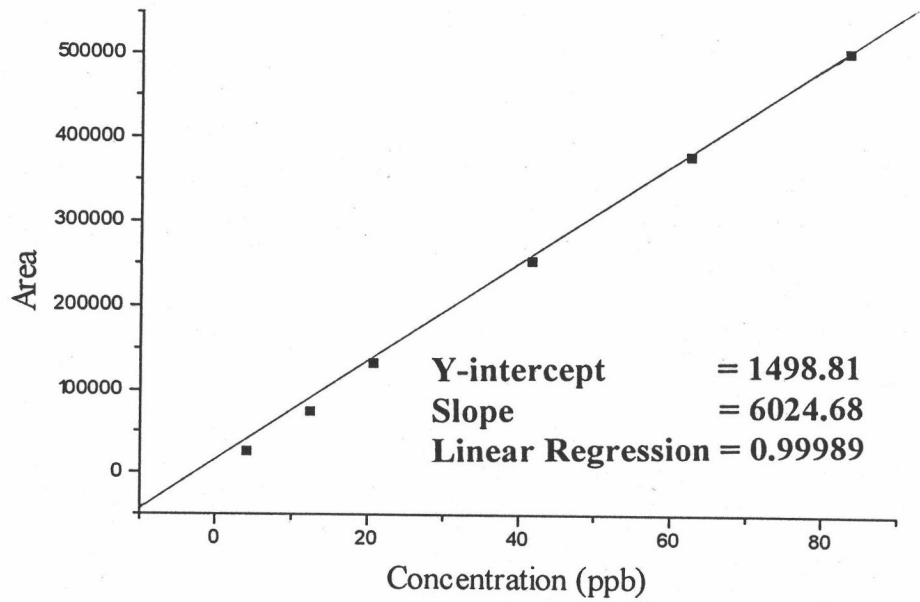


Figure 3.9 The calibration curve of standard DBP (high concentration) by the condition in Table 3.1

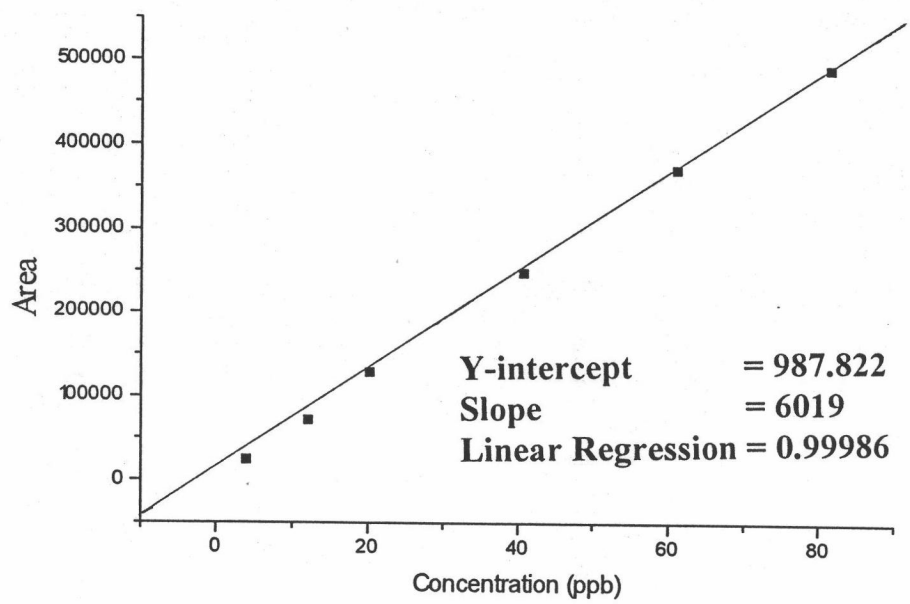


Figure 3.10 The calibration curve of standard BBP (high concentration) by the condition in Table 3.1

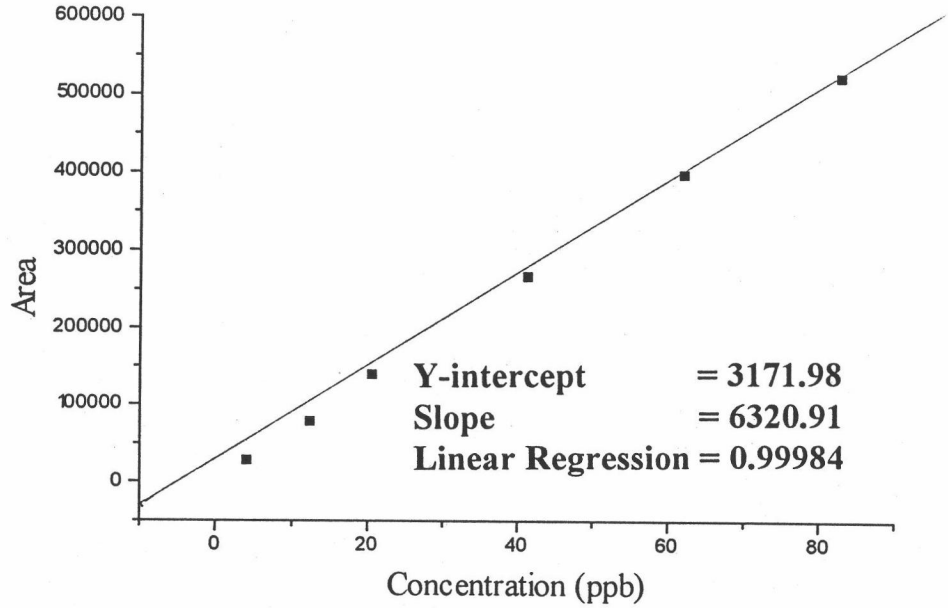


Figure 3.11 The calibration curve of standard DEHP (high concentration) by the condition in Table 3.1

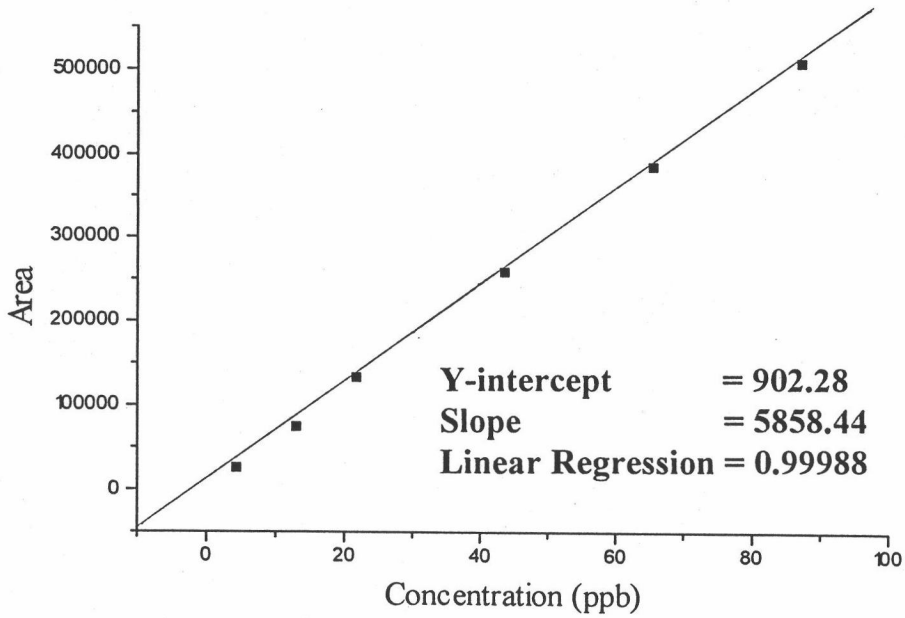


Figure 3.12 The calibration curve of standard DOP (high concentration) by the condition in Table 3.1

12. The Determination of Phthalate Esters in dairy products

Six dairy products from several supermarkets were purchased:

1. Yomost's yogurt from Seven eleven supermaket.

2. Yakult's milk from Mahboonkrong Sirichai supermaket.

3. Thai Denmark's orange yogurt milk from a glocery at Sam Yan, Patumwan, Thailand.

4. Yophalt's yogurt from Tokyu supermaket.

5. Meiji's yogurt from Tokyu supermaket.

6. Dutch Mill's yogurt from Isetan supermaket.

Each milk sample was prepared following the acidic adjustment milk sample (see 9.6)

1. Three 50.0000 g of each milk sample were adjusted to pH 2.10 by 10^{-2} M hydrochloric acid and 10^{-2} M sodium hydroxide.

2. The adjusted milk was then centrifuged at 3500 rpm for 10 min at 5°C.

3. The supernatant was pass through the disk in following steps.

13 Confirmation on the Structure of Some Phthalate Esters by GC/MSD

The extracts of milk samples from various supermarket in Bangkok by means of SPEM technique were confirmed by GC/MSD under GC/MSD condition in Table 3.3

Table 3.3 The GC/MSD conditions for the condition on structure of some PEs

GC Parameter	GC Condition
Analytical Column	24 m x 0.32 mm x 0.52 μm film thickness Ultra-1 (Crosslinked Methyl Silicone Gum) Capillary column
Temperature Program	A. 60°C (1 min) to 260°C (25°C/min) B. 260°C (6 min) to 280°C (5 min) (25°C/min)
Splitless time	1.0 min
Split Ratio	25 : 1
Flow Rate of: Carrier Gas(He)	2.00 mL/min
Detector	Mass Selective Detector
Inlet Temperature	280°C
Transfer line Temperature	280°C
Ion Source	EI (Electron-Impact) 70 eV
Ion Analyzer	Quandrupole