



# INDUSTRIAL TECHNOLOGY INSTITUTE (ITI)

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P. O. Box, 787, 363, Bauddhaloka Mawatha, Colombo 7, Sri Lanka.

Telephone: 0094 011 2379800 Fax: 0094 011 2379850

120/4 A, Vidya Mawatha, Colombo 7, Sri Lanka.

Telephone: 0094 011 2379800 Fax: 0094 011 2379950

CONFIDENTIAL

**ESTABLISHMENT OF RAPID HPLC METHOD  
TO DETECT DICYANADIAMIDE (DCD)  
IN MILK POWDER  
AND  
ANALYSIS RESULTS OF FEW SELECTED MILK POWDER SAMPLES**

Issued by

Chemical & Microbiological Laboratory  
Industrial Technology Institute

2013-07-26

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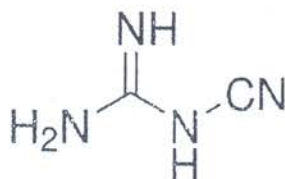
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## 0 Introduction

Dicyandamide (DCD, 2-Cyanoguanidine)  $C_2H_4N_4$  is a Nitrile compound derive from guanidine. Dicyandiamide (DCD) is used in pasture land to reduce greenhouse gas emissions and leaching of nitrogen into waterways. In early 2013, some reports surfaced identifying low levels of dicyandiamide in milk powders originating in New Zealand. While only small trace amounts of dicyandiamide were detected in the widely reported cases, high doses of DCD are considered toxic for humans. As a result of the finding, milk producers and government agencies moved quickly to reassure there was no risk to the public health. Need was arises to establish a method to detect and quantify DCD in milk powder imports to Sri Lanka.

Chemical Structure of Dicyandiamide:



Molecular Weight: 84.08

Appearance: White crystal

Density =  $1.400\text{g/cm}^3$

Solubility in water - 41.3 g/L

## 1. Scope

Determination of Dicyandiamide in milk powder by High Performance Liquid Chromatography with a UV - Detector

## 1. Method of Analysis

Approximately 1g of test sample was weighted into a screw capped vial and was dissolved in 1mL of de-ionized water. Then 2mL of acetonitrile was added and vortexed for 60 seconds. After separation of acetonitrile layer it was transferred into a MAS-quchERs cartridge. Extraction was repeated with another 2mL of acetonitrile. Combined acetonitrile layer in the MAS-quchERs cartridge was cleanedup by vortexing for 30 seconds and was centrifuged for 5min at 5500r/min. The supernatant was separated into another clean screw capped vial and was evaporated to dryness with slow nitrogen steam. Residue was dissolved in 1mL acetonitrile. The solution was filtered through 0.22  $\mu\text{m}$  membrane filter and 5 $\mu\text{L}$  of solution was injected into high pressure liquid chromatography (HLPC) system with UV detector (220nm). Concentration of DCD in milk powder samples were calculated by comparison of peak areas of DCD in test items with those of standards (calibration graph and equation was used).

ESTABLISHMENT OF RAPID HPLC METHOD TO DETECT DICYANDIAMIDE (DCD)  
IN  
MILK POWDER

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### 3.1 Standards preparation:

0.3mg/L, 0.5 mg/L, 1.0 mg/L and 2.0 mg/L DCD standard solutions were prepared by diluting the DCD 100 mg/L stock solution with de-ionized water.

### 3.2 Sample preparation

Different brands of milk powder samples (as given in following table 1) were prepared as per method 3.0.

Table 1- Brands of milk powder samples tested

Type of milk powder	Number of test samples prepared
Foreign Brand 1(Anchor-Fonterra)	10
Foreign Brand 2(Diamond)	02
Foreign Brand 3(Anchor 1+)	04
Foreign Brand 4(Maliban nonfat)	04
Local Brand 1(Palawatta)	05
Local Brand 2(Highland)	05

### 3.3 High Performance Liquid Chromatographic (HPLC) Condition:

Detector: Ultra Viloet (UV)

Column: Unisol Amide (HILIC)

Mobile Phase: Solvent A -10mM Ammonium Acetate (pH=4.0)

Solvent B - Acetonitrile

A: B = 15:85 (v/v)

Detector-UV 220 nm

Injection Volume: 5  $\mu$ L

Column Temperature: 29<sup>0</sup>C

Flow Rate: 0.8mL/min

### 4.0 Determination of Limit of Detection and Limit of Quantification

#### 4.1 Limit of Detection (LOD)

This is the lowest concentration of analyte in a sample that can be detected, but *necessarily quantitated, under the stated conditions of the test. When the measurements are made at low analyte levels, e.g. in trace analysis, it is important to know the concentration of the analyte that can be confidently detected by the method.*



# ESTABLISHMENT OF RAPID HPLC METHOD TO DETECT DICYANDIAMIDE (DCD)

## IN MILK POWDER

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### 4.2 Method of establishment of (LOD)

LOD was established with seven independent sample blanks fortified at lowest acceptable concentration (0.3mg/kg) of the analyte and analyzed for DCD on individual fortified samples.

$$\begin{aligned}\text{LOD} &= \text{Analyte concentration corresponding to a mean sample blank value} + 3s \text{ (definition)} \\ &= (\bar{x}) + 3s \\ &= 0.219 + 3 * 0.045 \\ &= 0.35 \text{ ppm} \\ &(\text{s}=\text{standard deviation, for } n=7)\end{aligned}$$

### 4.3 Limit of Quantification (LOQ)

Is the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated conditions of the analysis.

$$\begin{aligned}\text{LOQ} &= \text{Analyte concentration corresponding to a mean sample blank Value} + 6s \text{ (definition)} \\ &= (\bar{x}) + 6s \\ &= 0.219 + 6 * 0.045 \\ &= 0.5 \text{ ppm}\end{aligned}$$

### 5.0 Determination of Recovery

Analytical methods do not always measure all of the analyte of interest present in the sample. Therefore it is necessary to assess the efficiency of the method in detecting all of the analyte present.

#### 5.1 Establishment of Recovery

Known amount of DCD was spiked in three levels (0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg) to a matrix of milk powder with predetermined and verified DCD concentration (0.44 mg/kg). Then DCD of spiked samples were extracted as per the above method given in section 3 and quantified for DCD. Recovery percent was calculated as per the formula given in section 5.2 and results were given in table 2.

#### 5.2 Calculation of Recovery

$$\text{Recovery (\%)} = (C_1 - C_2) / C_3 * 100$$

Where  $C_1$  is the concentration determined in the fortified sample  
 $C_2$  = the concentration determined in unfortified sample  
 $C_3$  = concentration of fortification

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Table 2 – Results of Spike Recovery

Milk powder	DCD Concentration added (spiked) to sample (mg/kg)	DCD Concentration determined in Sample(mg/kg)	Determined DCD concentration (mg/kg)	Spiked Recovery %
Fonterra	0.5 (Low Level )	0.44	1.03	118
Fonterra	1.0 (Medium Level )	0.44	1.45	101
Fonterra	2.0 (High Level )	0.44	2.10	83

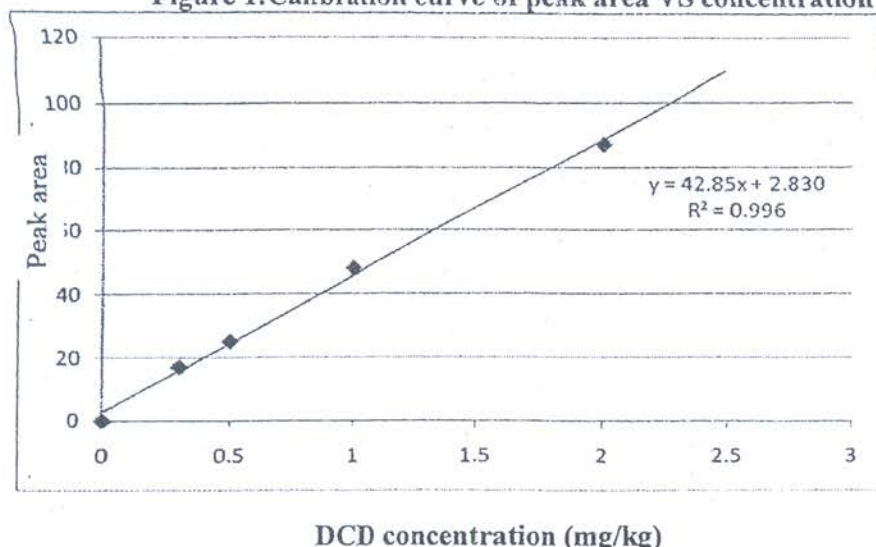
### 6.0 Linearity

The linearity of an analytical method is its ability to elicit test results that are (directly or by means of well-defined mathematical transformations) proportional to the concentration of analytes in samples within a given range. Linearity is determined by a series of three to six injections of five or more standards whose concentrations span 80-120 percent of the expected concentration range. The response should be (directly or by means of a well-defined mathematical calculation) proportional to the concentrations of the analytes.

### 6.1 Establishment of Linearity

The linearity of standard plot (Figure 1) was expressed in terms of the determination of coefficient ( $R^2$ ) from plot of the integrated peak area verses concentration of the DCD standard (mg/kg). The curve equation  $y = mx \pm c$  calculated with linear regression method, which was used to determine sample DCD concentrations. This equation was obtained over a range of concentration, in accordance with the levels of DCD found in milk powder.  $R^2$  value of the equation ( $y = mx \pm c$ ) of the curve was 0.997 shows the good linearity of the analytical method .

Figure 1: Calibration curve of peak area VS concentration for DCD





**7. Results and Discussion:-**

Summary of validation parameters is given in table 3.

Table 3 - Summary Results of Validation Parameters

Method Validation parameter	CML Result	Literature Results
Linearity (R <sup>2</sup> )	0.996	0.997
Recovery		
Low Level	118%	80%-90%
Mid Level	101%	
High Level	83%	
Limit of Detection (LOD)	0.35 mg/kg	
Limit of Quantification (LOQ)	0.5 mg/kg	0.5 mg/kg

Table 4 - Summary of DCD in tested milk powder samples

Brand of Milk Powder	DCD Concentration/mg/kg (Average)	DCD Concentration/mg/kg
Foreign Brand 1 (Fonterra)	0.64 (n=10)	Min- 0.36 Max-0.96
Foreign Brand 2 (Diamond)	0.67 (n=2)	Min- 0.65 Max-0.69
Foreign Brand 4 (Maliban N/F)	0.66 (n=4)	Min- 0.61 Max-0.72
Foreign Brand ( Anchor1+)	0.68 (n=4)	Min- 0.62 Max-.0.73
Local Brand 1(Highland)	Not Detected (n=5)	
Local Brand 2(Palawaththa)	Not Detected (n=5)	

n=number of replicates  
Max=Maximum concentration  
Min=Minimum concentration

N/F – Non Fat

*0.3 mg/kg  
acceptable level*

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**8.0 Conclusion:**

Dicyandiamide was detected in all analyzed foreign branded milk powder samples. Dicyandiamide was not detected in the tested local branded milk powder samples. The accuracy of the method cannot be determined due to unavailability of certified reference material (CRM) for DCD.

**9.0 Reference:**

HPLC-UV and HPLC-MS/MS published by Bonna –Agela Technologies,  
<http://www.agela.com/Download.aspx?id=557>



.....  
M.R.P. Dassanayake  
Research Scientist



.....  
P.S.F. Perera  
Research Scientist



.....  
J.K.A.B. Wijegunasekara  
Head- Chemical and Microbiological laboratory  
2013/07/26

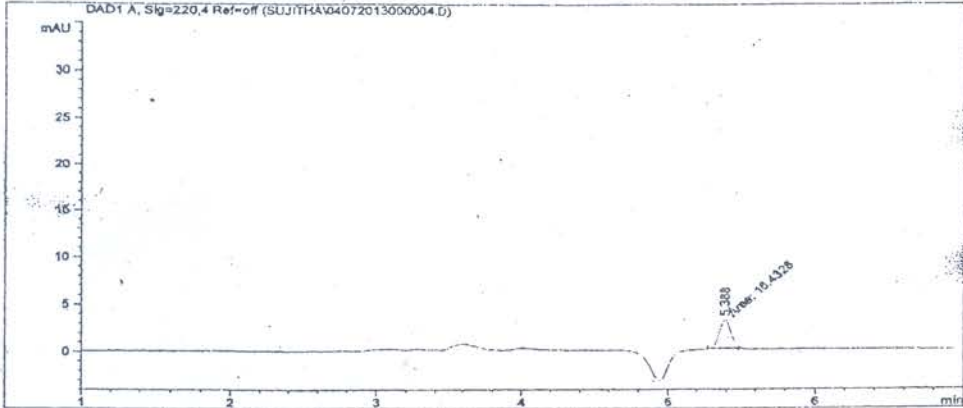
**J.K.A. Bandulasoma Wijegunasekara**  
Head  
Chemical & Microbiological Laboratory



## Chromatograms of Standards

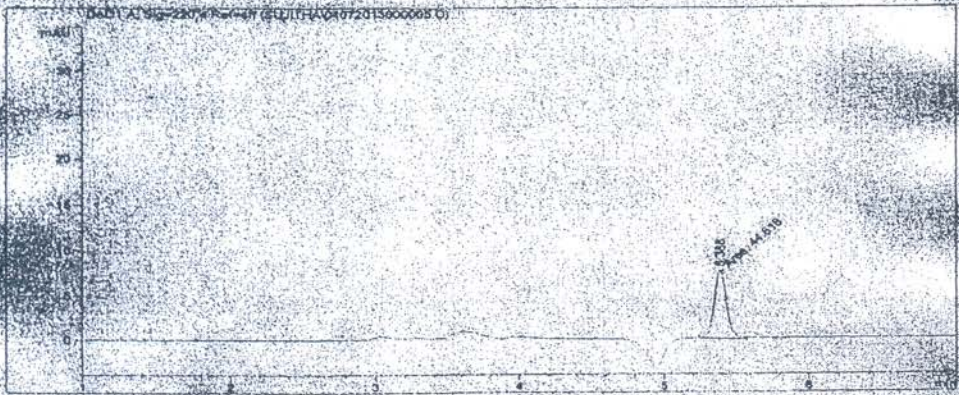
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 Acq. Instrument : Instrument 1 Location : Vial 4  
 Injection Date : 7/5/2013 10:22:16 AM Inj Volume : 5 µl  
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 Last changed : 7/5/2013 10:13:33 AM by Sujitha/ruwini  
 (modified after loading)  
 Analysis Method : C:\CHEM32\DCD.M  
 Last changed : 7/8/2013 3:32:28 PM by Sujitha/ruwini  
 (modified after loading)  
 Sample Info : Analysis of DCD



Data File C:\CHEM32\1\DATA\SUJITHA\04072013000005.D  
 Sample Name: DCD St 1.0 ppm -b

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 Acq. Operator : Sujitha/ruwini  
 Acq. Instrument : Instrument 1 Location : Vial 2  
 Injection Date : 7/5/2013 10:27:20 AM Inj Volume : 5 µl  
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 (modified after loading)  
 Analysis Method : C:\CHEM32\DCD.M  
 Last changed : 7/8/2013 3:32:28 PM by Sujitha/ruwini  
 (modified after loading)  
 Sample Info : Analysis of DCD



**Malibari**  
Non-Fat Milk Powder

Date of Manufacture கிடைத்த தேதி உற்பத்தித் திகதி	DD / MM / YY 20.11.2012	Maximum Retail Price LKR. கடமீ சிலை விலை அதிகபட்ச சிலை விலை	375.00
Date of Re-packing மறுபொதித்த தேதி மேல்தி செய்யப்பட்ட திகதி	15.03.2013 15.03.2014		13074A1
Date of Expiry காலாவதியாகும் தேதி காலாவதி திகதி		Batch Code காலாவதியை தொகுதிக் குறியீடு	

After opening pack, store contents in a cool, dry place.  
பிற்பாடு திறந்து வைக்கப்பட்ட பின்னர் குளிர், உலர் இடத்தில் வைக்கப்பட வேண்டும்.

**Anchor 1+**

Date of Manufacture கிடைத்த தேதி உற்பத்தித் திகதி	YY / MM / DD 12.10.13	Maximum Retail Price LKR. அதிகபட்ச சிலை விலை விலை ரூ.	410.00
Date of Re-packing மறுபொதித்த தேதி மேல்தி செய்யப்பட்ட திகதி	13.01.16/18.28 2014.01.16		107610163
Date of Expiry காலாவதியாகும் தேதி Date of Expiry		Batch Code காலாவதியை தொகுதிக் குறியீடு	

**Highland**  
FULL CREAM MILK POWDER

Date of Manufacture கிடைத்த தேதி உற்பத்தித் திகதி	DD / MM / YY 20.06.13	Maximum Retail Price LKR. அதிகபட்ச சிலை விலை விலை ரூ.	310.00
Date of Re-packing மறுபொதித்த தேதி மேல்தி செய்யப்பட்ட திகதி	01.06.13	Batch Code காலாவதியை தொகுதிக் குறியீடு	6252
Date of Expiry காலாவதியாகும் தேதி Expiry Date	20.06.14		

Anchor Full Cream

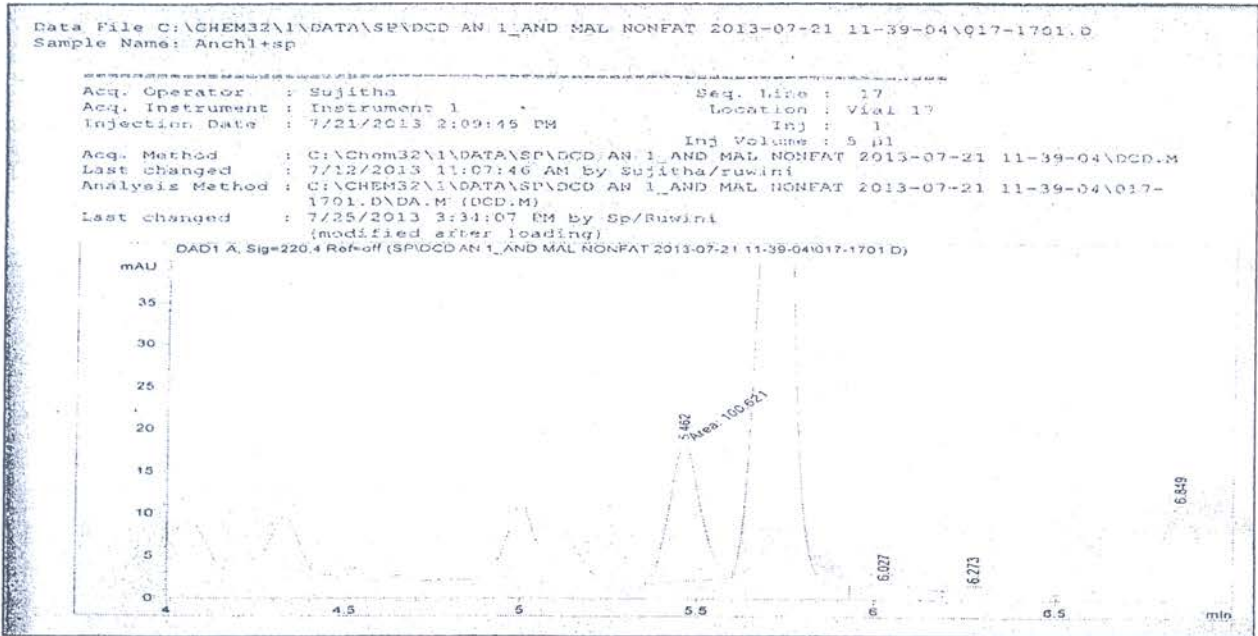
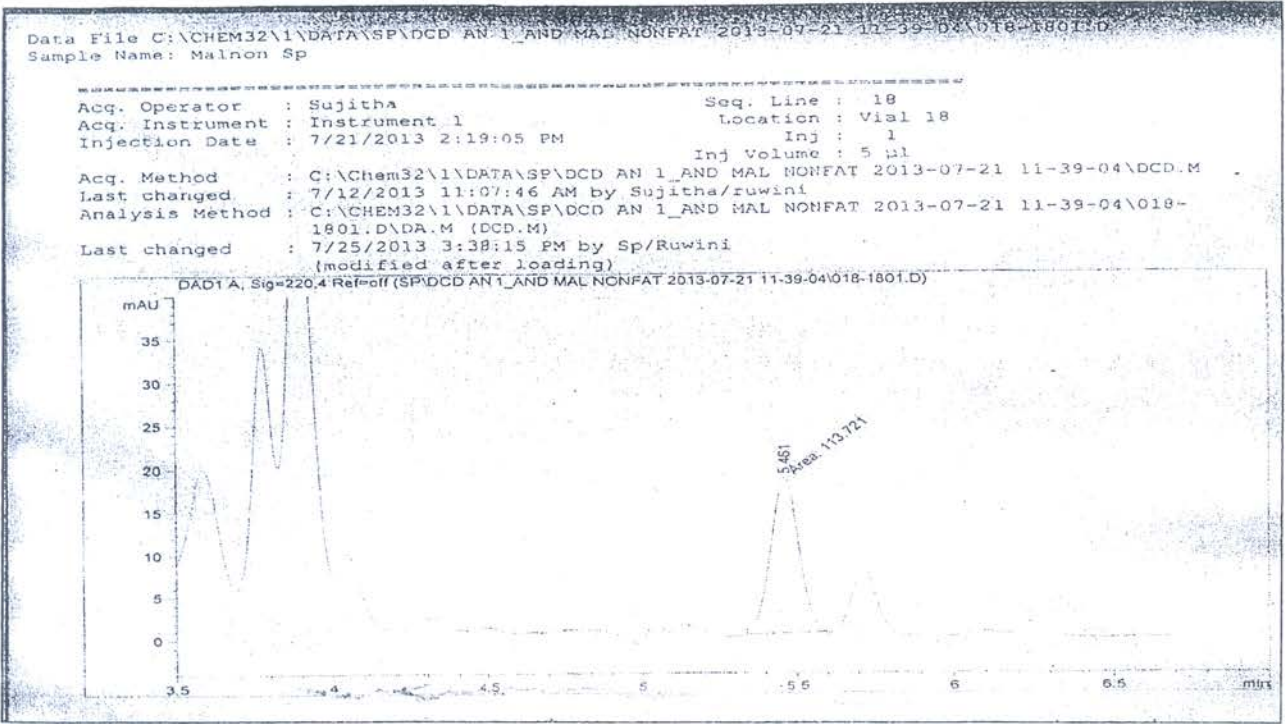
Batch No: 0605C0883  
11:21

2013/07/20

2013/7/26



Chromatograms of spiked sample



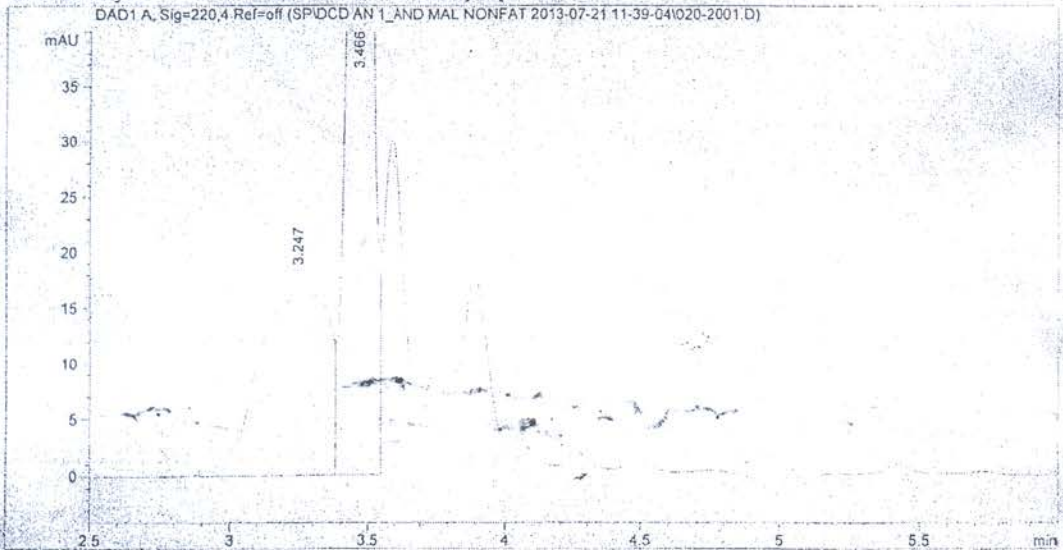
Spiked sample ch's

Chromatogram of Local branded milk powder

Data File C:\CHEM32\1\DATA\SPIDCD AN 1\_AND MAL NONFAT 2013-07-21 11-39-04\020-2001.D  
Sample Name: Hg1

=====

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	Inj Volume : 5 µl
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Last changed : 7/25/2013 3:38:51 PM by Sp/Ruwini	



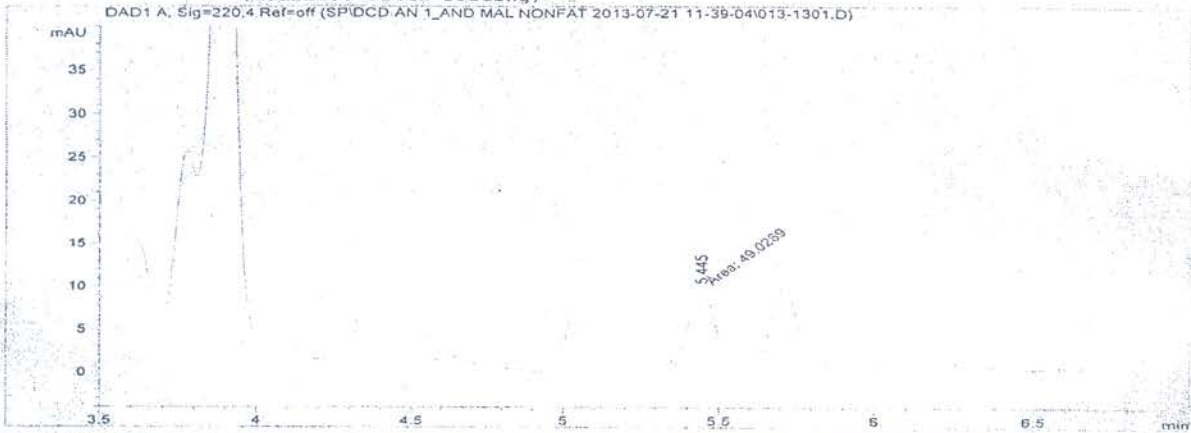
Highland (used as a blank)



Chromatograms of Foreign branded milk powder

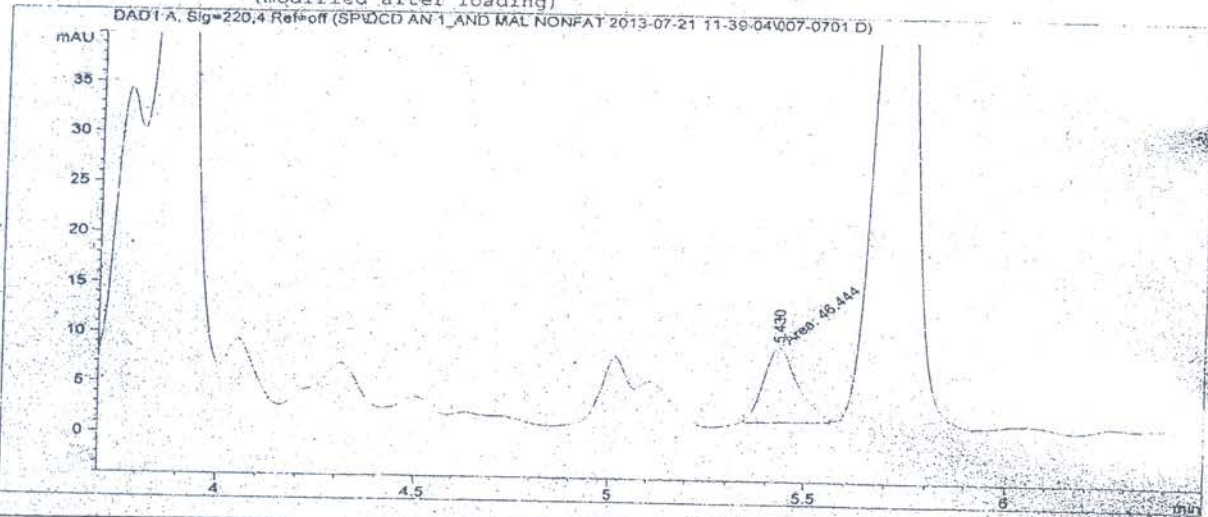
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 Sample Name: MalNon A

Acq. Operator : Sujitha Seq. Line : 13  
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 Injection Date : 7/21/2013 1:32:17 PM Inj : 1  
 Inj Volume : 5 µl  
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 Last changed : 7/26/2013 1:09:14 PM by Ruwini  
 (modified after loading)



ata File C:\CHEM32\1\DATA\SP\DCD AN 1 AND MAL NONFAT 2013-07-21 11-39-04\007-0701.D  
 Sample Name: Anchi+ A

Acq. Operator : Sujitha Seq. Line : 7  
 Acq. Instrument : Instrument 1 Location : Vial 7  
 Injection Date : 7/21/2013 12:35:55 PM Inj : 1  
 Inj Volume : 5 µl  
 Acq. Method : C:\Chem32\1\DATA\SP\DCD AN 1 AND MAL NONFAT 2013-07-21 11-39-04\DCD.M  
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 Analysis Method : C:\CHEM32\1\DATA\SP\DCD AN 1 AND MAL NONFAT 2013-07-21 11-39-04\007-0701.D\DA.M (DCD.M)  
 Last changed : 7/26/2013 1:20:32 PM by Ruwini  
 (modified after loading)



Maliban nonfat → 13074A1 (batch no)