HPTLC ANALYSIS OF THE EXCIPIENT- MELAMINE IN MARKTED MILK PRODUCTS

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Abstract

Aim: To develop and validate a sensitive, reliable and rapid High- Performance Thin Layer Chromatography method for the detection and quantification of Melamine in milk- based products.

Objectives: To Confirm the presence or absence of Melamine in milk products. To measure the concentration of Melamine in milk products and ensure it is within safe limits as per regulatory guidelines.To validate the method for its sensitivity and specificity.

Methodology: Milk Samples are collected from the market and deproteinized using 0.05ml of 37%v/v Hydrochloric acid solution and extracted using acetonitrile as a solvent .The extract is spotted onto a HPTLC Silica 60 F_{254} 10x10 standard plate against Melamine standard and developed using (Acetonitrile:Water:Ethyl acetate in the ratio of 3:1:1v/v/v) as a mobile phase. The plates are scanned under UV light at 257 nm and Melamine was quantified by comparing their R_f values and peak intensities with the standard and further FT-IR determination was also carried out for the confirmation of Melamine presence.

Result: Standard Melamine showed a distinct band at the expected R_f value, confirming the method Validity and in Markated formulation 1 Melamine was detectable but non-quantifiable but in Markated formulation 2 showed traces of Melamine higher than Markated formulation 1 but below the low limits quantification.

Conclusion: Markated formulation 2 and Markated formulation 1 both had trace amounts of Melamine that were below the quantifiable limits. The HPTLC method confirmed the absence of significant contamination.

Outcome: This study ensures that the samples are safe for consumption under regulatory limits and help manufacturers to avoid potential health risks associated with excessive Melamine contamination.

Keywords: Melamine, Milk, adulteration, food analysis, HPTLC, kidney damage

1. Introduction

Milk is a rich source of essential nutrients like protein, fat, carbohydrates, vitamins, and minerals for all age groups. However, milk adulteration remains a global concern due to factors like demand-supply gaps, its perishable nature, and limited detection methods. Common frauds, such as adding water or mixing milk from different species, are economically motivated and pose minor health risks. But harmful adulterants like urea, formalin, detergents, and melamine can have severe effects on human health (Kamthania et al., 2014). Melamine, a nitrogen-rich compound used in industries like plastics and fertilizers, is fraudulently added to milk and protein sources to falsely inflate protein content, exploiting nitrogen-based tests. This affects infants and children most due to their milk dependence and organ vulnerability. Melamine toxicity occurs when it combines with cyanuric acid in the body, forming crystals that damage renal cells, leading to kidney issues and even death. Incidents like the 2008 China milk scandal, causing over 50,000 infant hospitalizations, highlight its dangers. Regulatory limits for melamine in food have been set by Codex Alimentarius to ensure safety.

Melamine can enter milk and dairy products through various means, such as intentional adulteration to mimic protein, pesticide cyromazine metabolization in animals, and its presence in nitrogenous fertilizers or contaminated crops. It can also transfer from plastic packaging or melamine-fertilized pastures grazed by cows, with milk contamination detectable in as little as 8 hours. Dairy products show varying melamine levels, increasing in the order of milk, yogurt, coffee mate, cheese, and infant formula, posing serious health concerns Melamine ($C_3H_6N_6$) is a nitrogen-rich, slightly water-soluble, white crystalline compound used in producing plastics, coatings, adhesives, and containers. It forms a cross-linked polymer through a unique process involving methylol derivatives. Composed of 66% nitrogen, it releases nitrogen gas when burned, reducing its fire-retardant qualities. A metabolite of the pesticide cyromazine, melamine develops in mammals consuming cyromazine-treated crops. Discovered in 1834 by Justus Von Liebig, it is synthesized by heating diacyandiamide from calcium cyanamide.

HPTLC is the most powerful advanced form of TLC and consists of chromatographic layers of utmost separation efficiency and the application of sophisticated instrumentation for all steps in the procedure include accurate sample application, standardized reproducible chromatogram development and software controlled evaluation. HPTLC is a concept that includes a widely standardized methodology based on scientific facts as well as the use of validated methods for qualitative and quantitative analysis. HPTLC meets all quality requirements for today's analytical labs, to increase the resolution and to allow more accurate quantitative measurements.

2. Material and Methods

The required materials include chemicals like silica gel F_{254}, TLC plates, acetonitrile, ethyl

acetate, purified water, melamine (99% pure), and samples. The necessary glassware includes beakers, measuring cylinders, flasks, test tubes, funnels, a china dish, and pipettes of various sizes, along with a magnetic stirrer. Accessories such as non-absorbent cotton, filter paper, tripod stand, butter paper, and aluminum foil are also essential. For CAMAG devices, tools like the Automatic TLC Sampler 4 or Linomat 5, Automatic Developing Chamber ADC2 or Twin Trough Chamber, and TLC Scanner 3 with winCATS software are required for the procedures.

2.1 Collection of samples and standard

Samples were purchased from the market infant formulas such as **Marketed Formulation 1** is an infant formula manufactured by Nestlé, designed for growing children above 18 months. It contains essential nutrients like DHA, iron, and probiotics to support overall development and **Marketed Formulation 2** is a dairy-based milk powder produced by Amul, primarily used as a milk substitute for various purposes, including infant feeding when recommended and both the samples were collected based on the sales value in India. The standard Melamine is collected from Loba chemie PVT.LTD.

2.2 Thin layer chromatograpy:

TLC is a simple and effective method for detecting melamine in various samples, such as food and milk products was performed using Silica gel plate F254 Plates which is spotted with prepared Standard and Sample solutions. Mobile Phase is prepared using Acetonitrile:Ethanol:Water in the ratio of 3:1:1 V/V/V and saturated for 20 mins. After 20 mins ,plates were kept in the mobile phase chamber and developed. Then the plates were scanned by UV Scanner for the detection of spots.

2.3 UV-VISIBLE determination:

A Double beam UV-VIS spectrophotometer (Model: UV-1800, Shimadzu) was used to identify the melamine in milk powdered samples. The samples and standard were diluted with methanol and taken a quartz cell for the recording of the absorbance spectrum at wavelength between 220 to 260nm.

2.4 Fourier transform infrared (FT-IR) analysis

Fourier transform infrared spectroscopy makes the information about the functional groups determination present in the surface of samples in the form of stretching and bending frequencies of the molecules. The functional groups of samples were recorded from 400 to 4000cm-1 with a FT-IR Spectrophotometer at Drug Testing nanoparticles lab, Karpagam Academy of Higher Education, Coimbatore, India.

2.5 HPTLC-(High performance thin layer chromatography) Preparation of standard

10 mg of melamine are dissolved in 100 mL of water. 10 mL of this solution are diluted to 100 mL with methanol (concentration 0.01 mg/mL) and further serial dilutions were made for different concentrations and stored at 4c.

Preparation of Samples

5gm of both the powdered samples are mixed with 105 mL of methanol. Then 0.05 mL of HCl 37 % are added. After standing for 30 min the mixture is centrifuged for 5 min at RCF 2700. The supernatant is used as test solution.

2.6 Chromatographic analysis

HPTLC Silica gel 60 F_{254} plates in sizes 10 × 10 cm or 20 × 10 cm are used as the stationary phase. The test solution (1 or 2 µl) and standard solutions (0.5, 1, 2, 4, 6, 8, and 10 µl) are applied as 8 mm bands with a minimum spacing of 2 mm, positioned 8 mm from the lower edge of the plate. The developing solvent consists of a mixture of acetonitrile, water, and ethyl acetate in a 3:1:1 ratio. Development is carried out in a Twin Trough Chamber (10 × 10 cm or 20 × 10 cm) pre-saturated for 20 minutes with filter paper, using 10 mL of the mobile phase per trough. The plate is developed to a distance of 55 mm from the lower edge and then air-dried for 5 minutes using a cold air stream and further scanned by UV detector and FTIR analysis were carried out for functional characteristics

3. Result and Discussion:

3.1 Characterization of Melamine In Milk Powdered Samples by UV-VISIBLE Spectroscopy

The purchased Samples were dissolved in a suitable solvent and subjected to UV-VISIBLE Spectrophotometer and confirms the presence of melamine in it .The results through UV-VIS spectrum obtained for Sample-1 and Sample-2 are shown in the figure 1.a and 1.b.The peak obtained for the Samples varies in the range of 200-400nm which is identical to the characteristics of UV spectral analysis for Melamine .Various peaks obtained at 230nm, 234nm, 245nm, 247nm 256nm. At 230–240 nm there is Strong absorption due to $\pi \rightarrow \pi^*$ transitions in the triazine ring which indicates the presence of 6.2Melamine.

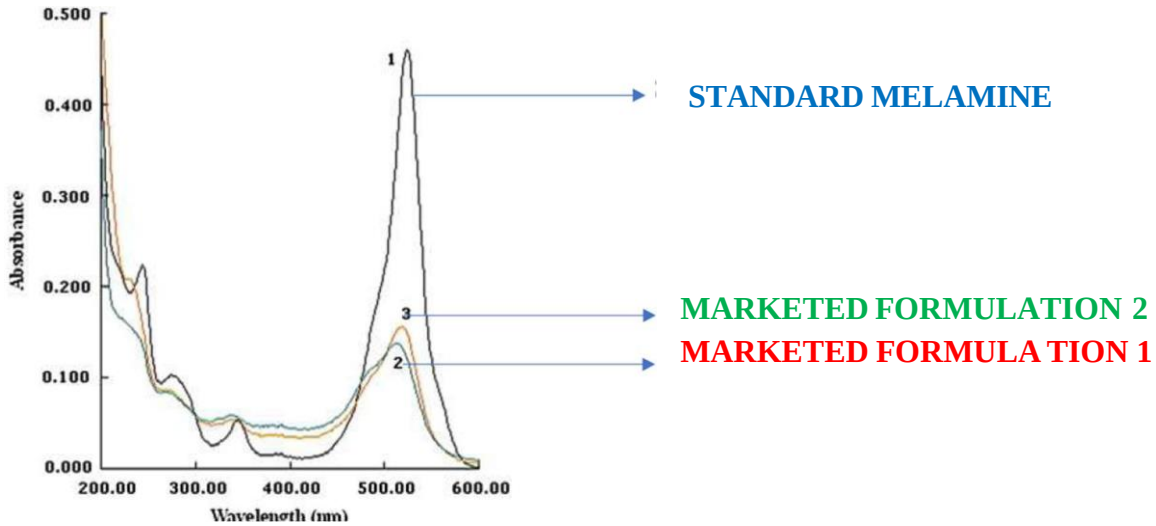

Figure 1: UV visible spectrum of Standard, Sample 1 , Sample 2

3.2 Fourier Transform Infrared (FT-IR) Analysis

FTIR spectroscopy is a useful technique for detecting melamine in milk powder by identifying its characteristic functional group vibrations. The presence of melamine is typically confirmed by its unique absorption bands in the mid-infrared (MIR) region (4000–400 cm^{-1}). Characteristic FT-IR Peaks of Melamine

3460–3400 cm^{-1} → N–H stretching (from primary amine groups) 3300–3100 cm^{-1} → N–H symmetric and asymmetric stretching 1650–1550 cm^{-1} → C=N stretching (triazine ring) 1460–1400 cm^{-1} → C–N stretching

810–750 cm^{-1} → Triazine ring breathing mode

FT-IR transmittance spectra of MEL standard and pure milk are shown. The IR transmission peaks at 3500– 3000 and 1700–1300 cm1 in the MEL spectrum are attributed to the stretching and bending vibrations of the amino groups present in the MEL. The characteristic band of the MEL standard was observed in the transmission spectrum

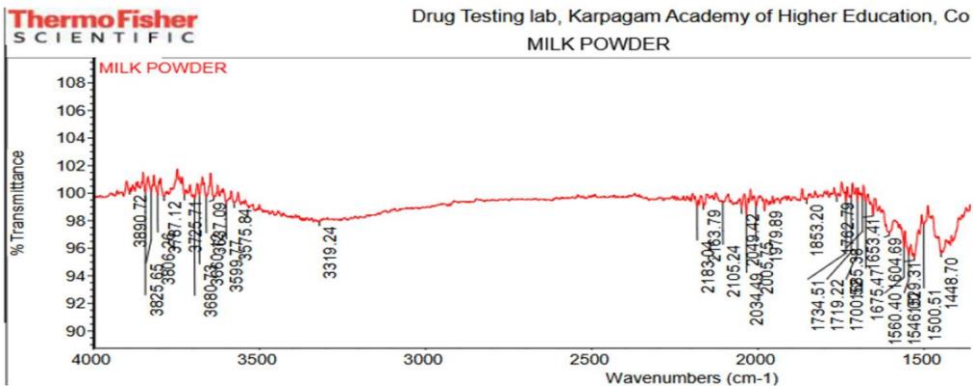

Figure 2: FT-IR ANALYSIS OF MILK PRODUCT SAMPLE

Spectrum : Milk Powder
Region : 3495.26-649.90 cm^{-1}
Search type : Correlation

TABLE 3: FT-IR interpretation

COMPOUND	**FREQUENCY**
Carboxyl(-COOH)	1700-1750 cm^{-1}
Amide 1 (-CONH - FROM PROTIENS)	1600-1700 cm^{-1}
Hydroxyl (-OH)	3200-3600 cm^{-1}
C-O-C GLYCOSIDE BOND IN LACTOSE	1700-1750 cm^{-1}
C-H (BENDING VIBRATION)	900-1250 cm^{-1}
Calcium	1300-1650 cm^{-1}
H-O-H	1600-1650 cm^{-1}

3.3 Determination of Melamine by HPTLC

The HPTLC analysis of powdered milk samples confirmed the presence of melamine, but its concentration was below the limit of quantification (LOQ). The obtained chromatographic profiles showed detectable spots corresponding to melamine, indicating trace amounts in the samples.

TABLE 4 : Plate setting for HPTLC

Stationary phase	TLC Silica F254, HPTLC SILICA GEL 60 F254
Plate format	100X100 mm
Application type	BAND
Application	Position y:8.0 mm, length :8.0 mm, width :0 mm
Track	1^{ST} POSTION X: 21.5mm, DISTANCE:11.4mm
Solvent front postion	70 mm

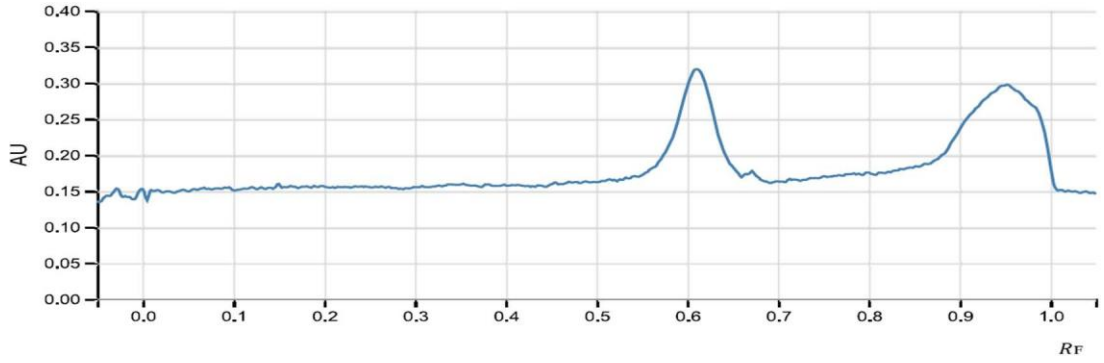

Figure 3: Melamine volume - 1.5 µL

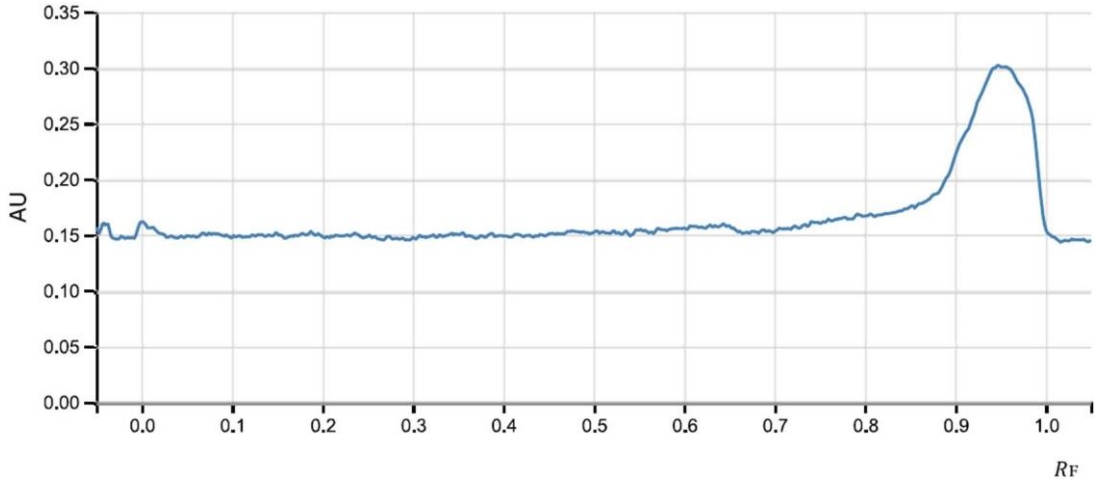

Figure 4: MARKETED FORMULATION 2 VOLUME - 1.5 µL

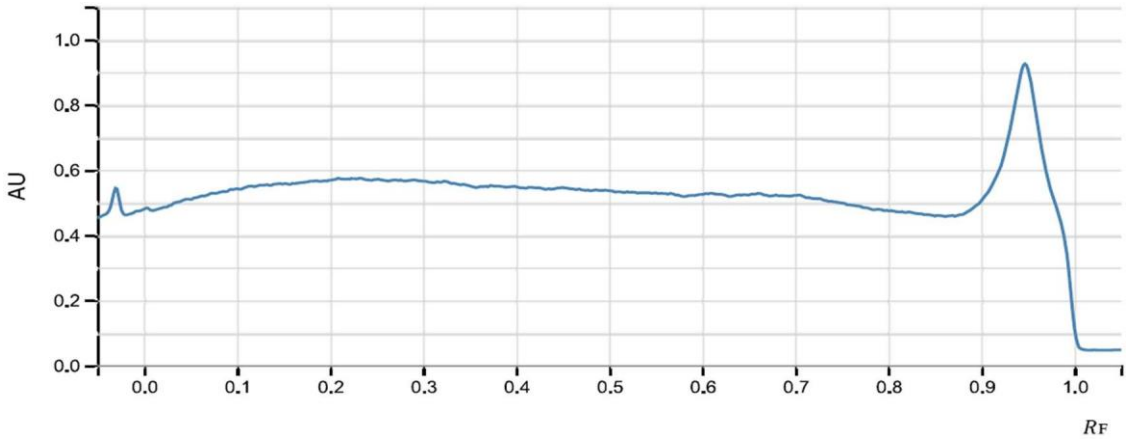

Figure 5: MARKETED FORMULATION 1 VOLUME - 1.5 µL

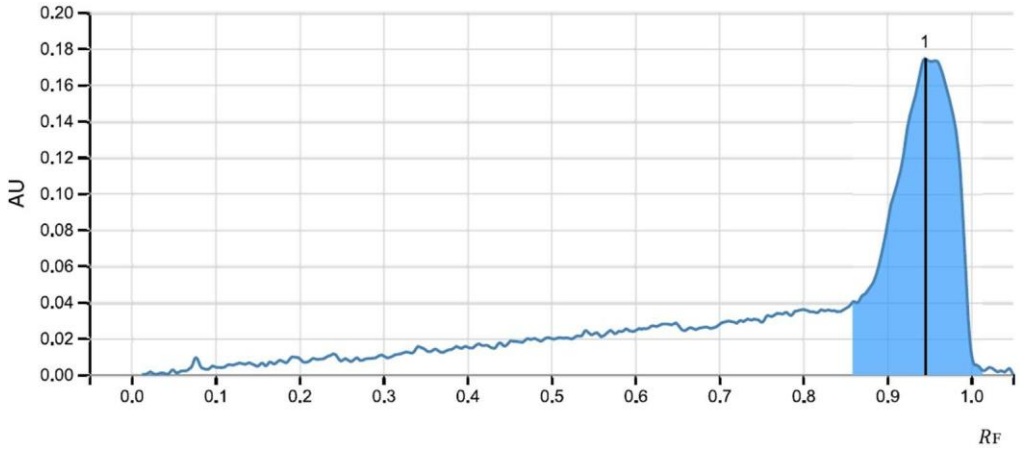

Figure 6 : MARKETED FORMULATION 1 VOLUME - 3.0 µL

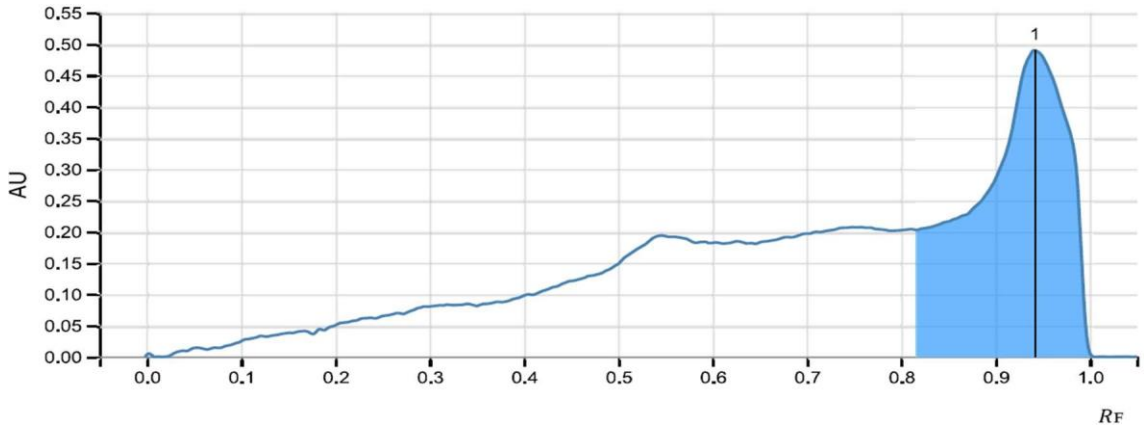

Figure 7 : MARKETED FORMULATION 2 VOLUME – 3.0 µL

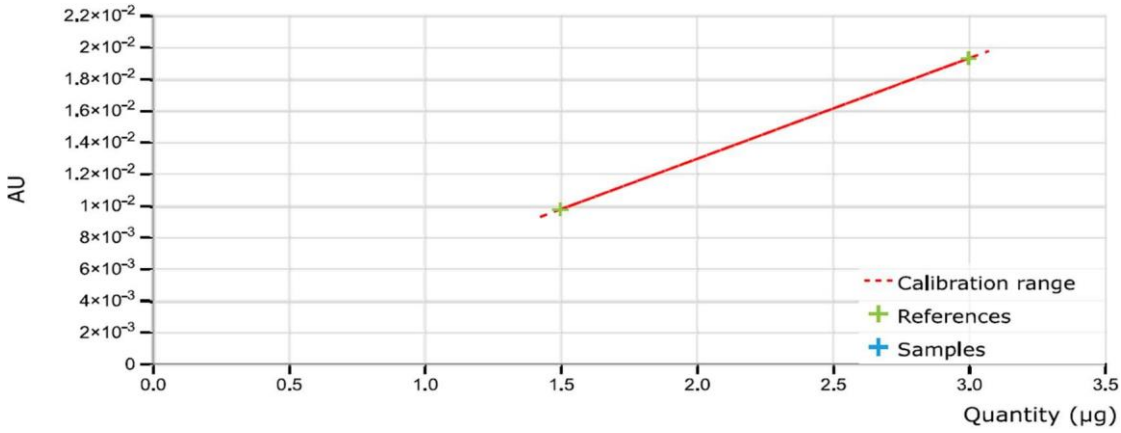

Figure 8: AREA CALIBRATION FOR SUBSTANCE MELAMINE @ 254nm

4. CONCLUSION:

Melamine contamination in infant formula poses a public health concern due to its toxic effects on kidney function, particularly in infants. It can enter food products during processing or from packaging materials. Regulatory agencies like the FDA, EFSA, and WHO have set safety limits, such as 1 ppm in infant formula. Techniques like UV-Visible absorption (200–400 nm) and FT-IR spectroscopy (amino group vibrations at 3500–3000 and 1700–1300 cm^{-1}) confirm melamine's presence. High Performance Thin Layer Chromatography detects trace amounts, with MARKETED formulation 2 showing slightly higher levels than Markated formulation 1but still below quantifiable limits. While trace contamination is unlikely to pose a health risk, it emphasizes the need for advanced detection methods. Continuous surveillance, GMP adherence, and public awareness are essential for reducing risks and ensuring infant safety. Research and regulatory vigilance remain critical to maintaining stringent food safety standards.

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