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# Detection and Evaluation of Synthetic Food Dyes in Ice Popsicles, Using Various Techniques

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A B S T R A C T

Food dyes are added to various food commodities to enhance their appearance. The food colorants then used were extracted mainly from natural sources, i.e., plant, insects, animals, and mineral. The use of synthetic food dyes increased dramatically after the industrial revolution in the late nineteenth century. And then, many food industrialists have been using artificial dyes to color in various food items. The reasons to use synthetic food colorants are to produce high tinctorial power and a wide array of shades as well as to correct natural variations in food colors that are lost during storage or processing of food. Apart from this, some food industrialists use synthetic food dye to hide the low quality and staled food products. Many synthetic food dyes like Sudan I-IV, amaranth, erythrosine, etc. were suspected of being toxic and were banned. Hence presently, the use of synthetic food dye is strictly regulated and each food dye requires Food and Drug Administration (FDA) approval before its inclusion in any food product. Considering this, the present study was planned to estimate the dyes used by vendors in ice popsicles and running a microbial analysis on the color samples as well as the ice used for the preparation of the same. The chemical characterization of these synthetic food dyes was done by using various analytical techniques, i.e., high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC). The samples were also tested for the presence of heavy metals. Chemical analysis revealed the presence of sunset yellow (E110), tartrazine (E102), brilliant blue (E133), azorubine (E122), fast green FCF (E143), and indigo carmine (E132). As both heavy metals, as well as food colorants, have been reported to be toxic, prolonged consumption of food products containing these dyes can cause adverse health effects in human beings. Therefore, the chemical as well as toxicological studies for the food products containing different food additives including the food dyes should be strictly implemented.



## Introduction

Food chemistry is deals with both biological and non-biological components of food products with their chemical processes and interactions [1]. Meat, poultry, lettuce, beer, and milk are a few examples of biological substances. It has many of the same components as biochemistry, such as carbohydrates, lipids, and protein, but it also requires water, vitamins, minerals, enzymes, and food additives [2]. This project, as the title suggests, deals with one topic of food chemistry, i.e., colors used in food products. It mainly involves the determination of synthetic food dyes (azo dyes) in ice popsicles and their quality for the consumption of this particular food item. Food dyes are added to various food commodities to enhance their appearance [3]. It is specifically added

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for sensory analysis purposes. People associate certain colors with certain flavors, and the color of food can influence the perceived flavor in anything from candy to wine [4]. Colour additives are used in foods for many reasons including [5]:

- To make food more attractive, appealing, appetizing, and informative
- Offset color loss due to exposure to light, air, temperature extremes, moisture, and storage conditions
- Correct natural variations in color
- Enhance colors that occur naturally
- Provide color to colorless and "fun" foods
- Allow consumers to identify products on sight, like candy flavors or medicine dosages [6]

The addition of dyes/colorants to food materials expectedly from around 1500 BC [7] where the candy makers used to add natural extracts and wine to improve the product's appearance [8]. During the time of the industrial revolution, analytical chemistry was primitive and the regulations few, which further gave rise to adulteration of food. Heavy metal and other inorganic element-containing compounds were cheap and used in foodstuff to restore the color [10]. Many color additives had never been tested for toxicity or other adverse effects. Historical records show that injuries, even deaths, resulted from tainted colorants. In 1851, about 200 people were poisoned in England, 17 of them fatally, directly as a result of eating adulterated lozenges [9]. In 1856, Mauveine, the first synthetic color, was developed by Sir William Henry Perkin, and by the turn of the century, unmonitored color additives were used all over Europe and the United States in all sorts of popular foods, including ketchup, mustard, jellies, and wine [11,12].

The use of these synthetic food dyes is one of the most controversial issues for the food industry when looked at from a health perspective [13]. Synthetic food dyes have been suspected of being toxic and many have been banned over the years [14]. Hence presently, synthetic food colors are more strictly regulated than at any other time in history and each food dye requires Food and Drug Administration (FDA) approval for use, before its inclusion in any type of food products [15]. In India, according to the Prevention of Food Adulteration Act [16] and the *Food Safety and Standards Authority of India (FSSAI, under the section Food Safety and Standards (Food Products Standard and Food Additives) Regulation 2011)*, 8 synthetic dyes viz., Brilliant blue FCF, Carmoisine/Azorubine, Erythrosine, Fast green FCF, Indigo carmine, Ponceau 4R, Sunset yellow FCF and Tartrazine are permitted to be used in the eatables but in a limited quantity i.e. 100 ppm max (singly or in combination) [17-18]. Unfortunately, many confectioners use very high concentrations of these synthetic food dyes without knowing their toxic effects (a mixture of about 200ppm concentration is generally used) [19].

The main characteristics of synthetic food dyes are:

- The Colour is uniform.
- Highly stable to light, oxygen, and pH.
- Inexpensive.
- Harmful to humans if exceed 0.2 g/kg of food [20-22]

Some studies have reported the use of textile and non-permitted dyes in food items which have the potential to cause risks to human health [23,24]. The permitted food dyes are commonly available in the market in form of blends of two or more dyes and are widely encountered in a variety of food products from both urban and rural market [25-27]. The effect of these blends can be additive, synergistic, potentiating, or even antagonistic [28-30]. Majority of synthetic food dyes can lead to various kinds of allergic reactions like eczema, skin dermatoses [31], affect the liver, [28-32] lungs, [33] reproductive system [34,28]and immune system [35]. The number of food dyes added by confectioners to the drinks and other food commodities generally exceeds the authorized limit [36]. Hence monitoring of the levels of food dyes in highly consumed products such as beverages, processed foods, snacks, and desserts becomes mandatory. [37-43]. There are various methods known for the determination of the chemical composition of synthetic food dyes in soft drinks, juices, jellies, and other food commodities.

These methods include:

- Thin Layer Chromatography (TLC) [44,45]
- High-Performance Liquid Chromatography (HPLC) with Ultraviolet/Visible (UV/Vis) and Diode-array detector (DAD) [46]
- Liquid Chromatography-Mass Spectrometry (LC-MS) [47,48]
- Toxicological assays.
- Electrochemistry [49,50]
- Ion-pair chromatography [51]
- Spectrophotometry [52,54]
- Capillary Electrophoresis [55,56]

From the above-mentioned methods for determination, the first two were implemented in this project work. All of these synthetic dyes, along with some non-permitted colors are used in ice popsicles, which is a very common liquid beverage consumed by a majority of the Indian population, especially in a hot climate. It is also prepared under extremely unhygienic conditions, and hence microbiological assay was performed, for certain micro-organisms under this project work, along with the quality of the water/ice used for the preparation of the ice popsicle, popularly known as ice popsicles.

The microbial assay was done for the confirmation of the presence of 4 different organisms including *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi* and *Escherichia Coli*. Heavy metal testing was performed on two samples to investigate the presence of lead and cadmium.

**Microbial Analysis**

Bacteriological analysis is a method of analyzing liquids to estimate the numbers of bacteria present and, if needed, to find out what sort of bacteria they are. It represents one aspect of quality. It is a microbiological analytical procedure that uses samples of a particular liquid and from these samples determines the concentration of bacteria. The most reliable methods are the direct plate count method and membrane filtration method. mEndo Agar is used in the membrane filtration while VRBA Agar is used in the direct plate count method. VRBA stands for violet red bile agar. A media that contains bile salts which promote the growth of gram-negative and have the inhibitory characteristic to gram-positive although not completely inhibitory. These media contain lactose which is usually fermented by lactose fermenting bacteria producing colonies that can be identified and characterized. Lactose fermenting produces colored colonies while non-lactose fermenting produces colorless ones. Because the analysis is always based on a very small sample taken from a very large volume, all methods rely on statistical principles [57].

The method that is utilized under this project is the plate count method. The plate count method relies on bacteria growing a colony on a nutrient medium so that the colony becomes visible to the naked eye. The laboratory procedure involves making serial dilutions of the sample (1:10, 1:100, 1:1000, etc.) in sterile water and cultivating these on a nutrient agar plate that is sealed and incubated. Typical media include MacConkey agar to count gram-negative bacteria such as *Escherichia coli*. Typically one set of plates is incubated at 22°C and for 24 hours and a second set at 37°C for 24 hours. The composition of the nutrient usually includes reagents that resist the growth of non-target organisms and make the target organism easily identified, often by a color change in the medium [58,59]. Under this project, microbial analysis of the samples is performed for four different organisms, including:

1. *Escherichia coli* (MacConkey agar plates were used to detect the presence of this particular microorganism).
2. *Pseudomonas aeruginosa* (Cetrimide agar plates were used to detect the presence of the microorganism).
3. *Salmonella typhi* (Xylose Lysine Deoxycholate agar plates were used to detect the presence of the microorganism).
4. *Staphylococcus aureus* (Mannitol salt agar plates were used to detect the presence of the microorganism).

**Most Probable Number (MPN)**

Most Probable Number (MPN) is a method used to estimate the con-

centration of viable microorganisms in a sample through replicate liquid broth growth in ten-fold dilutions. MPN is most commonly applied for quality testing of water, i.e., to ensure whether the water is safe or not in terms of bacteria present in it. Under this project, MPN is performed on the water samples collected from the vendors that use this water in preparation for ice popsicles.

**HPLC Analysis**

In analytical chemistry, high-performance liquid chromatography (also known as high-pressure liquid chromatography) is a method for identifying, separating and quantifying each element in a mixture. It relies on pumps to transport a pressurised liquid solvent containing the sample mixture through a solid adsorbent material-filled column. Column which is filled with adsorbent material. It has been used for manufacturing legal (e.g., detecting performance enhancement drugs present in urine), (e.g., during the production of pharmaceutical and biological products), research (e.g., separating the components of a complex biological sample), and medical (e.g., detecting vitamin D levels in blood serum) purposes [60].

The synthetic dyes that were found to be present in the collected samples include the following:

Tartrazine. (azo dye)

Azorubine. (azo dye)

Fast green FCF.

Indigo carmine. (azo dye)

Sunset Yellow FCF. (azo dye)

Brilliant Blue FCF.

**TLC Analysis**

TLC stands for thin-layer chromatography, and is a chromatography method for separating and identifying non-volatile mixtures [61]. Thin-layer chromatography is achieved on a glass, plastic, or aluminium foil sheet that has been coated with a thin layer of adsorbent material, typically silica gel or aluminium oxide (alumina). This adsorbent layer is known as the stationary phase. A solvent mixture (mobile phase) is drawn up the plate through capillary action after the sample has been added to the plate. Because different analytes move up the TLC plate at different rates, separation is achieved [62]. For quantification of the results, the distance travelled by the substance being considered is split by the total distance travelled by the mobile phase. This ratio is called the Retardation Factor (Rf). In general, one that has a similar structure to the mobile phase will have a high retardation factor, while a substance whose structure resembles the stationary phase will have low Rf. The characteristics of retardation factors vary depending on the state of the stationary and mobile phases. For this reason, a sample of a known compound is applied to the plate before running the experiment.

**Heavy Metal Analysis**

Humans are exposed to several "heavy metals" such as mercury, cadmium and its organic form methyl mercury, uranium, lead & other metals as well as metalloids, such as arsenic, in the environment, workplace, water supply and food. Exposure to these metals may have adverse health effects. The main threats to human health from heavy metals are associated with exposure to cadmium, mercury, lead and arsenic (arsenic is a metalloid, but is usually classified as heavy metal). Heavy metals are defined as metallic elements that have a relatively high density compared to water [63]. Although heavy metals are naturally occurring elements that are found throughout the earth's crust, most environmental contamination and human exposure result from anthropogenic activities such as mining and smelting operations, domestic and agricultural use of metals, industrial production and use, metal-containing compounds [64-67]. It has been reported that metals such as cobalt (Co), nickel (Ni), selenium (Se) and zinc (Zn), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo) are essential nutrients that are required for Physiological & biochemical functions. Inadequate supply of these micro-nutrients results in a variety of deficiency diseases or syndromes [68]. Heavy metals are also considered as trace elements because of their presence in trace concentrations (ppb range to less than 10ppm) in various environmental matrices [69]. In biological systems, heavy metals have been reported to affect cellular organelles and components such as endoplasmic reticulum, cell membrane, nuclei, mitochondrial, lysosomes & some enzymes involved in metabolism, detoxification, damage repair [70]. Cadmium is a heavy metal of considerable environmental & occupational concern. It is widely distributed in the earth's crust at an average concentration of about 0.1 mg/kg [71]. Cadmium is a severe pulmonary and gastrointestinal irritant, which can be fatal if inhaled or ingested. After acute ingestion, symptoms such as abdominal pain, burning sensation, vomiting, salivation, muscle cramps, nausea, shock, loss of consciousness, vertigo and convulsions usually appear within 15 to 30 min [72]. Cadmium is classified by IARC as carcinogenic to humans and causes Lung cancer [73]. Lead is a naturally occurring bluish-grey metal present in small amounts in the earth's crust [74]. Acute exposure to lead induces brain damage, kidney damage, and gastrointestinal diseases, while chronic exposure may cause adverse effects on the blood, kidneys, central nervous system, blood pressure, and vitamin D metabolism [75-82].

## MATERIALS AND METHODS

**HPLC Analysis**

*HPLC was performed at ADARSH SCIENTIFIC RESEARCH CENTER & TESTING LAB PVT.LTD using the SOP prepared by the same for liquid beverages*. HPLC analysis was done for four different color samples, including red, orange, green, and yellow. STANDARD OPERATING PROCEDURE (SOP) for determination of artificial food colors in beverages was as follows:

**Purpose:** To describe the method for the determination of Artificial colors in beverages and food products by using HPLC with a UV detector.

**Scope:** This SOP is applicable for the determination of artificial colors including Tartrazine, Azorubine, Fast green, Sunset Yellow, Brilliant Blue, Indigo Carmine, and Brilliant blue by extracting the sample by water and Quantified the concentration by using UV /DAD detector.

**Procedure**

**Requirements:**

- HPLC with UV Detector
- Centrifuge (capable of providing 8000 rpm)
- Vortex Mixer
- Calibrated volumetric Flasks (50 mL, 25 mL, 10 mL)
- Calibrated Pipettes (1, 5, 10, 25 mL)
- Analytical balance
- Ultrasonic Bath
- Calibrated Micropipette (20 – 200$\mu$L & 100 – 1000 $\mu$L)

**Consumables**

- Reagent water (Milli-Q-water)
- Methanol, HPLC Grade
- Dipotassium hydrogen phosphate anhydrous
- Phosphoric acid

**Sample Preparation for Carbonated Beverages**

- It is necessary to get a representative sample for analysis so homogenize the sample properly by using mortar and pestle and proceed for sample preparation.
- Take 12.5 gm of the crushed sample into a 25 ml volumetric flask and add 20 ml of water mix well or sonicate it for at least 15 min.
- Make up the volume, and filter the supernatant through a 0.45$\mu$m syringe filter and Inject.

**Mobile Phase Preparation**

- Mobile phase A: Buffer solution: Weigh 3.4856g of Dipotassium hydrogen phosphate in 1 liter of the volumetric flask, add 950 ml of water and dissolve it completely and adjust the pH of the solution to 7.00 with phosphoric acid and make up the volume.
- Mobile phase B: Methanol

**Standard Preparation:** Take 10 mg of individual sample in 10 mL of

volumetric flask and makeup to the mark, with water.

**Preparation of calibration curve:** Prepare the following concentrations ranging from 10 to 100 mg/L from the stock solution and label them properly.

Diluent Used: - Water.

The calibration curve will be prepared before analysis.

The Linearity range may vary depending on the concentration of analyte quantified.

**Table 1:** Sample Preparation for the Calibration Curve.

| Standard. | Stock Solution. | 100ppm Standard Mixture. | 75ppm Standard Mixture. | 50ppm Standard Mixture. | 25ppm Standard Mixture. | 10ppm Standard Mixture. | 5ppm Standard Mixture. |
|---|---|---|---|---|---|---|---|
| Tartrazine. | 1000 mg/kg | 100 µl from each individual sample and make up to 1 ml. | 75 µl from each individual sample and make up to 1 ml. | 50 µl from each individual sample and make up to 1 ml. | 25 µl from each individual sample and make up to 1 ml. | 10 µl from each individual sample and make up to 1 ml. | 5 µl from each individual sample and make up to 1 ml. |
| Azorubine. | | | | | | | |
| Fast green FCF. | | | | | | | |
| Indigo carmine. | | | | | | | |
| Sunset yellow FCF. | | | | | | | |
| Brilliant blue FCF. | | | | | | | |

7) HPLC Conditions:

- Flow rate: 1.0mL/min
- Injection volume: 20µL
- Wavelength: 254 nm
- Column temperature: 30°C
- Column: C18 250mm X 4.6mm X 5µm
- Mobile phase: Gradient as mentioned in the table given below.
- Run time: 20 min

| Time (minutes) | Mobile phase A | Mobile phase B |
|---|---|---|
| 0.5 | 90 | 10 |
| 11 | 40 | 60 |
| 18 | 90 | 10 |

**Microbial Analysis**

**Aim:** Detection of Microorganism in Food Dyes used in Ice Popsicles

**Procedure:** Sample preparation for a test of specified micro-organisms:

For a test of Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Salmonella prepare the sample using not less than 1 g or 1 ml of sample or 1 in 10 dilution corresponding to not less than 1 g or 1 ml of sample to be examined and dissolve or dilute in 100 ml sterile Soybean Casein Digest Medium (SCDM) and incubate at 30 to 35°C for 18 to 24hours.

**Test for *Staphylococcus aureus*:**

- After incubation of Soybean Casein Digest Medium (Prepared above), mix Soybean Casein Digest Medium tube and streak loopful on a plate of Mannitol salt agar (MSA) using inoculating loop.
- Invert the plate and incubate at 30°C to 35°C for 18 to 72 hours.
- After incubation, examine the plates and the possible presence of Staphylococcus aureus is indicated by the growth of yellow or white colonies surrounded by a yellow zone.
- If colonies are found, a further identification test (Catalase test) was performed.

  In this test, the colonies were collected with the aid of a loop and mixed with 5ml Hydrogen peroxide (6%) and 5ml Distilled water. If effervescence was observed, the presence of *Staphylococcus aureus* is confirmed.
- If there are no colonies of the type described observed or if the identification tests are negative, the product complies with the test for Staphylococcus aureus.
- Record the results.

**Test for *Pseudomonas aeruginosa*:**

- After incubation of Soybean Casein Digest Medium (Prepared above), mix Soybean Casein Digest Medium tube and streak loopful on a plate of Cetrimide Agar using inoculating loop.
- Invert the plate and incubate at 30°C to 35°C for 18 to 72 hours.
- After incubation, examine the plate and growth of colonies indicates the possible presence of *Pseudomonas aeruginosa*.
- If there are no colonies observed, the product complies with the test for *Pseudomonas aeruginosa*.
- Record the results.

**Test for *Salmonella typhi*:**

- After incubation of Soybean Casein Digest Medium (Prepared above), mix the Soybean Casein Digest Medium tube and transfer 0.1 ml using a sterile pipette to 10 ml of Salmonella Enrichment Broth (SEB) and incubate at 30°C to 35°C for 18 to 24 hours.
- After incubation, mix the contents of the tube and streak a loopful from SEB media on a plate of Xylose lysine Deoxycholate agar (XLD) media using an inoculating loop.
- Invert the plate and incubate at 30°C to 35°C for 18 to 48 hours.
- After incubation, examine the plate and the possible presence of Salmonella is indicated by the growth of well-developed, red colonies, with or without black centers.
- If there are no colonies of the type described observed, the product complies with the test for Salmonella.
- Record the results.

**Test for *Escherichia coli*:**

- After incubation of Soybean Casein Digest Medium (Prepared above) mix the Soybean Casein Digest Medium tube and transfer 1.0 ml using a sterile pipette to a tube containing 100 ml of MacConkey's broth and Incubate at 42°C to 44°C for 24 to

48 hours.

- After incubation, mix the contents of the tube and streak a loopful from

MacConkey's broth media on a plate of MacConkey's agar media using an inoculating loop and incubate at 30°C to 35°C for 18-72 hours in an inverted position.

- The growth of colonies indicates the possible presence of Escherichia coli.
- If colonies are found, a further identification test with tryptone water was performed, by observing the pink ring at the junction of the liquid.
- If there are no colonies observed or if the identification tests are negative, the product complies with the test for Escherichia coli.
- Record the results.

**Observation Table**

**Table 2:** Observations after microbial analysis.

| S. No. | Location | Sample | Microorganism | Observation |
|---|---|---|---|---|
| **Red Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | - |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Orange Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| 1. | Seawoods. | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Yellow Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | + |
| **Green Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | - |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Red Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | - |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Orange Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | - |
| | | iii) | *Staphylococcus aureus* | + |
| 2. | Dadar. | iv) | *Pseudomonas aeruginosa* | - |
| **Yellow Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | - |
| | | | | |
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Green Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | - |
| | | iii) | *Staphylococcus aureus* | - |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Red Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | - |

| | | | | |
|---|---|---|---|---|
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Orange Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | - |
| 3. | Prabhadevi. | iv) | *Pseudomonas aeruginosa* | - |
| | | | | |
| | | | **Yellow Sample** | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Green Sample** | | | | |
| | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Red Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Orange** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | - |
| | | iii) | *Staphylococcus aureus* | + |
| 4. | Nerul. | iv) | *Pseudomonas aeruginosa* | - |
| **Yellow Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | + |
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |
| **Green Sample** | | | | |
| | | i) | *Escherichia coli* | + |
| | | ii) | *Salmonella typhi* | - |
| | | iii) | *Staphylococcus aureus* | + |
| | | iv) | *Pseudomonas aeruginosa* | - |

**Most Probable Number (MPN)**

**Aim:** To determine the quality of water by multiple tube fermentation test (Most Probable Number, MPN).

**Requirements:**

- Medium: Lactose broth
- Glasswares: Test tubes, Durham tube
- Others: Sterile pipettes

**Preparation of medium:**

- Prepare medium (Lactose broth) in single and double strength concentration.
- Dispense the double strength medium in 3 tubes (10ml in each tube) and single strength medium in 6 tubes (10 ml). Examine the tubes to make sure that the inner vial is full of liquid with no air bubbles.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. (l in each tube) and add a Durham tube in an inverted position.

**Procedure:**

- Take 3 tubes of double strength and 6 tubes of single strength for each water sample to be tested.
- Using a sterile pipette add 10 ml of water to 3 tubes containing 10 ml double strength medium.
- Similarly, add 1 ml of water to 3 tubes containing 10 ml single strength medium and 0.1 ml water to the remaining 3 tubes containing 10 ml single strength medium.
- Incubate all the tubes at 37°C for 24 hrs. If no tubes appear positive re-incubate up to 48 hrs.
- Compare the number of tubes giving positive reaction to a standard chart and record the number of bacteria present in it.

**Confirmatory test:** Tryptone water test:

- Incubate the tryptone water at (44.5 ±0.2°C) for 18-24 hours
- Following incubation, add approximately 0.1ml of Kovacs reagent and mix gently.
- The presence of indole is indicated by a red color in the Kovacs reagent, forming a film over the aqueous phase of the medium.

1. Confirmatory tests positive for indole, growth, and gas production show the presence of thermotolerant *E. coli*.
2. Growth and gas production in the absence of indole confirm thermotolerant coliforms.

Since some of the positive results from the confirmatory test may be false, it is desirable to do completed tests. For this inoculum from each positive tube of the confirmatory test is streaked on a plate of EMB or Endo agar. In this process, a loopful of the sample from each positive tubes is streaked onto a selective medium like Eosin Methylene Blue agar or Endo's medium. One plate each is incubated at 37°C and another at 44.5± 0.2°C for 24 hours. Following incubation, all plates are examined for the presence of typical colonies. Coliforms produce colonies with a greenish metallic sheen which differentiates them from non-coliform colonies (show no sheen). The presence of typical colonies at high temperatures (44.5±0.2) indicates the presence of thermotolerant *E. coli*.

**Observation Table:**

**Table 3:** observation of the first Sample 1

| SR NO. | MEDIA. | SAMPLE QUANTITY. | TEST TUBE NO. | ACID/GAS PRODUCTION. | POSITIVE TEST TUBES. | MPN/100ml |
|---|---|---|---|---|---|---|
| 1. | DOUBLE STRENGTH. | 10ml | 1 | + | 3 | >1100 |
| | | | 2 | + | | |
| | | | 3 | + | | |
| 2. | SINGLE STRENGTH. | 1ml | 1 | + | 3 | |
| | | | 2 | + | | |
| | | | 3 | + | | |
| 3. | SINGLE STRENGTH. | 0.1ml | 1 | + | 3 | |
| | | | 2 | + | | |
| | | | 3 | + | | |

**Table 4:** observation of the second Sample 2

| SR NO. | MEDIA. | SAMPLE QUANTITY. | TEST TUBE NO. | ACID/GAS PRODUCTION. | POSITIVE TEST TUBES. | MPN/100ml |
|---|---|---|---|---|---|---|
| 1. | DOUBLE STRENGTH. | 10ml | 1 | + | 3 | 23 |
| | | | 2 | + | | |
| | | | 3 | + | | |
| 2. | SINGLE STRENGTH. | 1ml | 1 | - | 0 | |
| | | | 2 | - | | |
| | | | 3 | - | | |
| 3. | SINGLE STRENGTH. | 0.1ml | 1 | - | 0 | |
| | | | 2 | - | | |
| | | | 3 | - | | |

**TLC Analysis**

**Aim:** To perform thin layer chromatography of the extracted color dyes from the samples.

**Material and Equipment:**

- TLC plate. Size of plate- 5 cm x 20 cm, 20 cm x 20 cm ,10cm x 20 cm
- Filter paper.
- Capillary for spotting.
- Saturation chamber (beaker).
- Petri dishes.

**Chemicals Required:**

**Mobile phase:** Methanol: Toluene (11:9) v/v.

Color samples: Red, Yellow, Orange, Green. Procedure:

1) Preparing the developing chamber: The developing container for TLC can be a specially designed chamber, a jar with a lid, or a beaker with a watch glass/ Petri plate on the top. Pour solvent (Mobile phase) into the chamber to a depth of just less than 0.5cm.
2) Preparation of TLC plate.

Glass plates or flexible plates are commonly used for spreading the adsorbent.

Size of plate- 5 cm x 20 cm, 20 cm x 20 cm ,10cm x 20 cm.

Silica plates are most commonly used for TLC.

3) Spotting the TLC plate.

Place the narrow end of the capillary into a vial containing a solution (Colour sample) of the substance to be analyzed. Allow the solution to rising in the capillary. Hold the capillary above the TLC plate. Leave the capillary in contact with the plate for a brief amount of time, so that, the spot is not larger than 1mm in diameter.

4) Developing the TLC plate.

Place the prepared TLC plate in the developing chamber, cover the beaker with a Petri plate, and leave it undisturbed. The solvent rises the TLC plate by capillary action making sure the solvent does not cover the spot.

Remove the plate from the beaker and immediately mark the solvent front with a pencil. Allow the plate to dry.

5) Visualization of the TLC plate.

If there are any colored spots, circle them lightly with a pencil.

6) Measurement of Rf:

Rf = Distance traveled by solute

Distance traveled by the solvent

**Heavy metal analysis**

*The heavy metal analysis was performed at ADARSH SCIENTIFIC RESEARCH CENTER & TESTING LAB PVT.LTD.*

It was performed for 2 different color samples, including red and green.

The two samples were tested for the presence of Cadmium and Lead.

**Observation Table:**

**Table 5:** observation for heavy metal analysis

| Solution label | Cd (214.439nm) | Pb (220.353nm) |
|---|---|---|
| Green. | 0.70 (ppm) | 1.81 (ppm) |
| Red. | 0.80 (ppm) | 3.33 (ppm) |

**RESULTS**

**Results obtained after HPLC analysis**

**Table 6:** Result of HPLC Analysis

| S. No. | Sample | Standard Concentration (maximum ppm, singly or in combination) | Concentration of synthetic dyes found in each sample after HPLC analysis |
|---|---|---|---|
| 1. | Orange | 100 ppm | 191.731 ppm |
| 2. | Red | 100 ppm | 171.114 ppm |
| 3. | Yellow | 100 ppm | 130.849 ppm |
| 4. | Green | 100 ppm | 88.028 ppm |

As per the results obtained after HPLC analysis, it could be concluded that only one sample complied with the specification of the standard concentration, and the rest of the samples exceeded the maximum concentration of 100 ppm.

The orange sample included synthetic dyes in the concentration

of 191.731 ppm (sunset yellow), which exceeded 100 ppm the most. The red sample included synthetic dyes in the concentration of 171.114 ppm (sunset yellow, azorubine) which had the second-highest amount of dyes present out of the four samples tested. The yellow sample included synthetic dyes in the concentration of 130.849 ppm (tartrazine), which again exceeded 100 ppm. The Green sample was the only one that complied with the specification, as it included synthetic dyes in the concentration of 88.028 ppm (tartrazine, azorubine, brilliant blue). Orange, red, and yellow samples were proved to be not safe for consumption, while the concentration of dyes in the green sample was found to be under the limit. The red and the green sample were also found to contain more than one synthetic dye, indicating contamination.

**Result obtained after microbial analysis**

According to the observations based on microbial analysis mentioned in Table 2, Red color sample collected from Seawoods gives i) and ii) positive, Orange sample gives i), ii) and iii) positive, Yellow sample gives i), ii), iii) and iv) positive, the Green sample gives i) and ii) positive.

Red color sample collected from Dadar gives i) and ii) positive, Orange sample gives i) and iii) positive, Yellow sample gives i) and iii) positive, the Green sample gives i) positive.

Red color sample collected from Prabhadevi gives i) and iii) positive, Orange sample gives i) and ii) positive, Yellow sample gives i), ii) and iii) positive, the Green sample gives i), ii) and iii) positive.

Red color sample collected from Nerul gives i), ii) and iii) positive, Orange sample gives i) and iii) positive, Yellow sample gives i), ii) and iii) positive, the Green sample gives i) and iii), positive.

Where,

i. *Escherichia coli,*
ii. *Salmonella typhi,*
iii. *Staphylococcus aureus, and*
iv. *Pseudomonas aeruginosa.*

The study was conducted to determine the presence or absence of microorganisms in collected samples. According to the microbial test, it was detected that 9 out 16 test for Red samples were positive, 9 out 16 test for Orange samples were positive, 12 out 16 test for Yellow samples were positive, 8 out 16 test for Green samples were positive, which implies that the majority of the samples contain the previously mentioned microorganisms.

**Effects of *Escherichia coli* on the human body:**

*Escherichia coli* is a bacterium generally found in the gut of warm-blooded animals. Most strains of this bacterium are not harmful, however, some types can cause illness in humans, including diarrhea, abdominal pain, fever, and sometimes vomiting. It can also lead to kidney failure in sensitive patients. Few strains of this bacterium produce a toxin known as Shiga, which can cause an intestinal infection.

This bacterium is found to be present in all the samples checked for microbial growth, hence the food item should be avoided.

**Results were obtained after the MPN method.**

As mentioned in Table 3 and Table 4, both the samples analyzed for MPN contain bacteria/microorganisms and are unfit for consumption by human beings. The first sample was found to contain more than 1100 MPN/100 ml, where both single strength and double strength tubes exhibited acid production hence increasing the most probable number. Whereas the second sample was found to contain 23 MPN/100 ml, as only the double strength tubes exhibited acid production, and the single strength tubes were negative.

**Results obtained after TLC analysis**

**Table 7:** TLC- rf values of colored samples

| S.No | Sample | Std Rf values | Sample Rf value |
|---|---|---|---|
| 1. | Red | 0.7 | 0.43 |
| 2. | Yellow | 0.42 | 0.166 |
| 3. | Orange | 0.8 | 0.433 |
| 4. | Green | 0.5 | 0.14 |

As mentioned in Table 7, the standard RF values do not match the Rf values of individual samples, indicating there must be impurities present in the sample, other than the components present in the standard synthetic dyes. The polarity of the components present in both, the sample and the standard solution, also differs as inferred by the difference in the Rf values of the two.

**Results obtained after Heavy Metal analysis.**

As mentioned in Table 5, the two samples analyzed for the presence of two heavy metals, including Cadmium and Lead, show the presence of the heavy metals. Cadmium (Cd) was found to be present in the concentration of 0.70 ppm in the green sample, and in the concentration of 0.80 ppm in the red sample. Lead (Pb) was found to be present in a higher concentration as compared to cadmium. The green sample was found to contain Pb in the concentration of 1.81 ppm, whereas the red sample was found to contain Pb in the concentration of 3.33 ppm.

**Conclusion**

From this study, HPLC analysis revealed the presence of the already mentioned dyes. The concentration of these permitted dyes was NOT found to be within limits, i.e., 100ppm singly or in combination, except in the green sample. The standard and sample Rf values in TLC were found to be different. Therefore, it can be conclude that the sample is impure. Microbiological analysis performed, exhibited the presence of at least one pathogen per sample, from each location. Heavy metal analysis revealed the presence of lead in a higher quantity as compared to cadmium. Toxicity of synthetic food dyes containing a -N=N- moiety was discussed upon.

**Conflict of Interest**

Author not declare any conflict of interest

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**SUPPLIMENTORY DATA**

**HPLC Analysis of Orange Sample**

Sample ID-ASL-2020-27

Data-D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\ASL-2020-27

Method name- D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\Artificial Colour.met

Injection Volume - 20$\mu$l

Vial position - P1-A8

Acquired - 2/6/2020 4:25:55 PM (GMT +05:30)

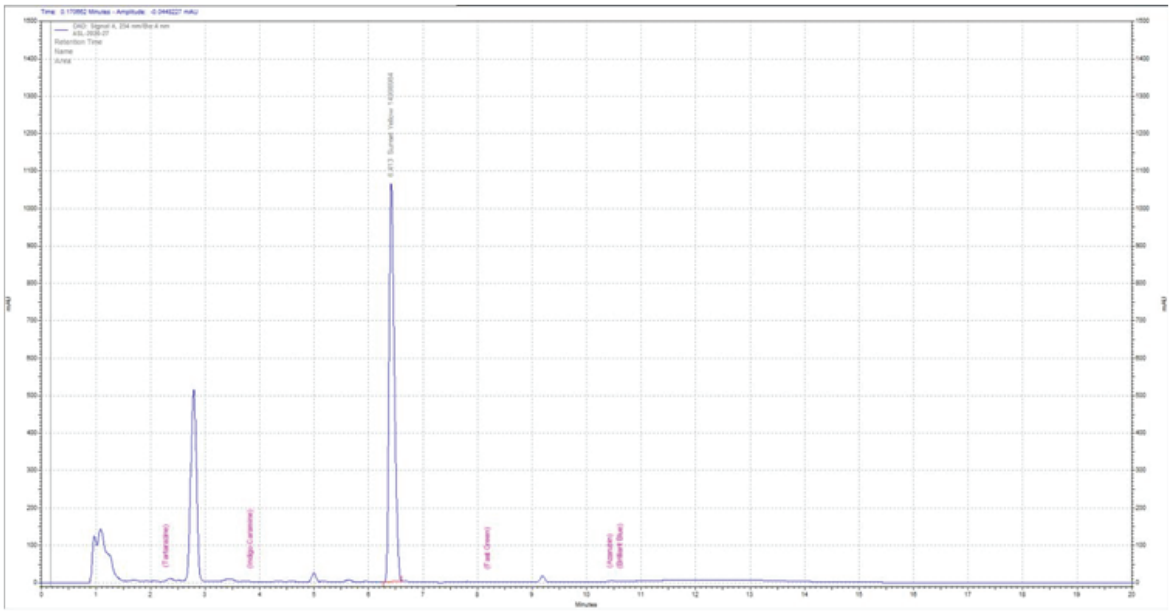

**Figure 1:** HPLC Analysis of Orange Sample

DAD: Signal A, 254 nm/Bw:4 nm

**Table 1:** HPLC Data from the Orange Sample

| Observation | | | | | |
|---|---|---|---|---|---|
| **Peak No.** | **Name** | **Retention Time** | **Area** | **Concentration** | **Units** (mg/l) |
| 1 | Tartrazine | 6.413 | 14998984 | 0.000 BDL | |
| | Indigo Carmine | | | 0.000 BDL | |
| | Sunset Yellow | | | 191.731 | |
| | Fast Green | | | 0.000 BDL | |
| | Azorubine | | | 0.000 BDL | |
| | Brilliant Blue | | | 0.000 BDL | |
| | | | | | |
| Totals | | | | | |
| | | | | 191.731 | |

**HPLC ANALYSIS OF YELLOW SAMPLE**

Sample ID-ASL-2020-28

Data-D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\ASL-2020-28

Method Name-D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\Artificial Colour.met

Injection Vol.- 20$\mu$l

Vial Position- P1-A9

Acquired-2/6/2020 4:48:08 PM (GMT +05:30)

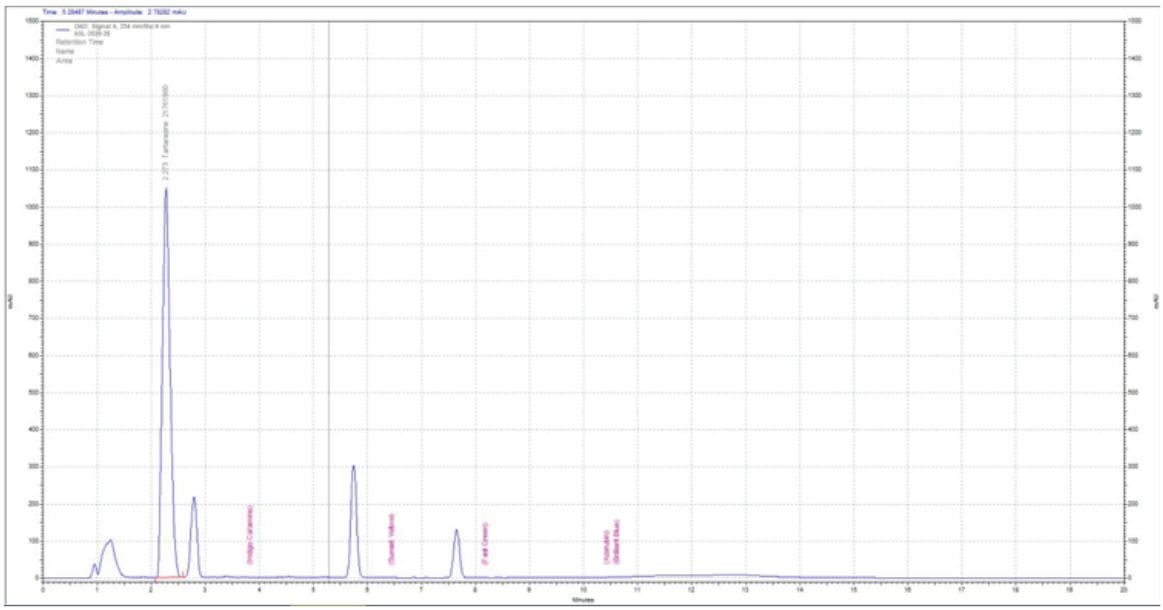

**Figure 2:** HPLC analysis of Yellow Sample

DAD: Signal A, 254 nm/Bw:4 nm

**Table 2:** HPLC Data from the Yellow Sample

| Observation- | | | | | | |
|---|---|---|---|---|---|---|
| **Peak No.** | | **Name** | **Retention Time** | **Area** | **Concentration** | **Units** |
| 1 | | Tartrazine | 2.273 | 21761960 | 130.849 | mg/l |
| | | Indigo Carmine | | | 0.000 BDL | |
| | | Sunset Yellow | | | 0.000 BDL | |
| | | Fast Green | | | 0.000 BDL | |
| | | Azorubine | | | 0.000 BDL | |
| | | Brilliant Blue | | | 0.000 BDL | |
| | | | | | | |
| Totals | | | | | | |
| | | | | 21761960 | 130.849 | |

**HPLC ANALYSIS OF RED SAMPLE**

Sample ID-ASL-2020-29

Data-D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\ASL-2020-29

Method Name-D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\Artificial Colour.met

Injection Vol.: 20$\mu$l

Vial Position- P1-A10

Acquired: 2/6/2020 5:10:21 PM (GMT +05:30)

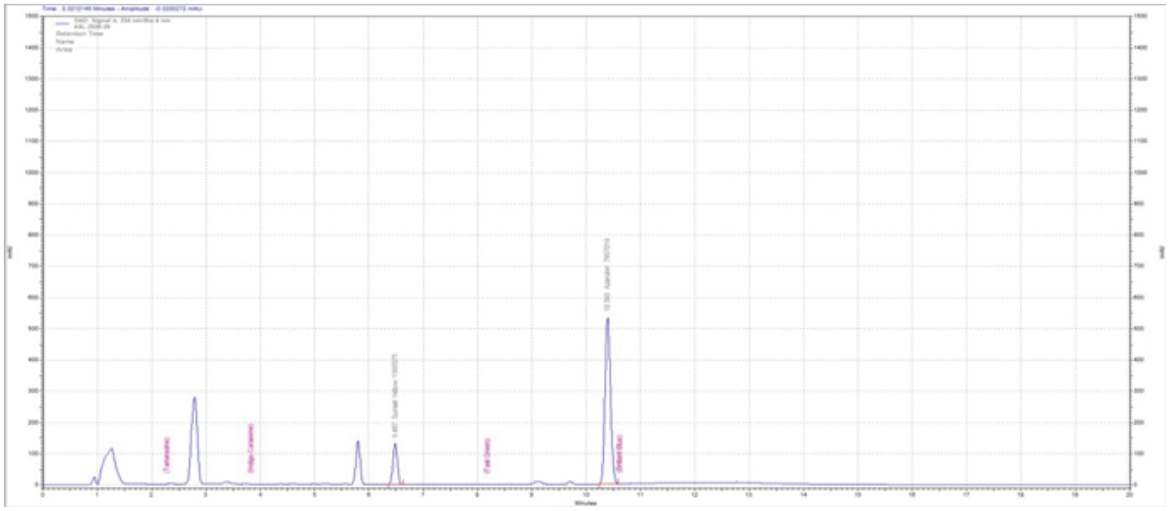

**Table 3:** HPLC Data from the Red Sample.

| **DAD: Signal A, 254 nm/Bw:4 nm** | | | | | |
|---|---|---|---|---|---|
| **Observation-** | | | | | |
| Peak No. | Name | Retention Time | Area | Concentration | Units |
| | Tartrazine | | | 0.000 BDL | mg/l |
| | Indigo Carmine | | | 0.000 BDL | |
| 1 | Sunset Yellow | 6.487 | 1565575 | 19.847 | |
| | Fast Green | | | 0.000 BDL | |
| 2 | Azorubine | 10.393 | 7857019 | 151.267 | |
| | Brilliant Blue | | | 0.000 BDL | |
| | | | | | |
| Totals | | | | | |
| | | | 9422594 | 171.114 | |

**HPLC ANALYSIS OF GREEN SAMPLE**

Sample ID-ASL-2020-30

Data- D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\ASL-2020-30

Method Name-D:\HPLC Data\HPLC DAD\Result\2020\Feb-2020\06-02-2020-Artificial Food Colour\06-02-2020-Artificial Food Colour.rslt\Artificial Colour.met

Injection Vol.- 20$\mu$l

Vial Position- P1-A11

Acquired-2/6/2020 5:32:36 PM (GMT +05:30)

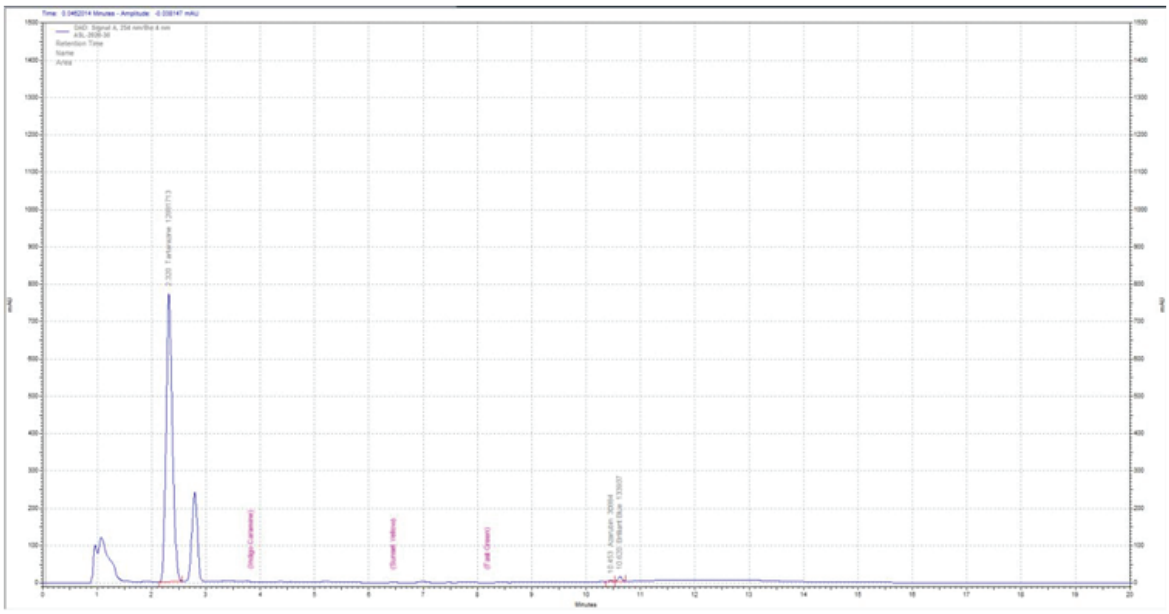

Figure 4: HPLC analysis of green sample

DAD: Signal A,254 nm/Bw:4nm

**Table 4:** HPLC Data from the Green Sample

| Peak No. | Name | Retention Time | Area | Concentration | Units |
|---|---|---|---|---|---|
| 1 | Tartrazine | 2.320 | 12881713 | 77.389 | mg/l |
| | Indigo | | | 0.000 BDL | |
| | Carmine | | | | |
| | Sunset Yellow | | | 0.000 BDL | |
| | Fast Green | | | 0.000 BDL | |
| 2 | Azorubine | 10.453 | 30884 | 0.059 | |
| 3 | Brilliant Blue | 10.620 | 133937 | 10.580 | |
| | | | | | |
| Totals | | | | | |
| | | | 13046534 | 88.028 | |