ANALYSIS OF CAFFEINE, THEOBROMINE, VANILLIN, AND PHENYLETHYLAMINE IN COMMON BEVERAGES AND FOOD PRODUCTS BY LIQUID CHROMATOGRAPHY

A Dissertation Report for

Course code and Course Title: CGO-500 & Dissertation

Credits: 8

Submitted in partial fulfilment of Master's Degree (MSc) in Analytical Chemistry

by

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Seal of the School

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Analysis of Caffeine, Theobromine, Vanillin and Phenylethylamine in Common Beverages and Food Products by Liquid Chromatography**" is based on the results of investigations carried out by me in the Analytical Chemistry at the School of Chemical Sciences, Goa University under the supervision of Dr. Prajesh S. Volvoikar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Analysis of Caffeine, Theobromine, Vanillin and Phenylethylamine in Common Beverages and Food Products by Liquid Chromatography"** is a bonafide work carried out by **Ms. Sheetal Sharma** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Discipline Chemistry at the School of Chemical Sciences, Goa University.

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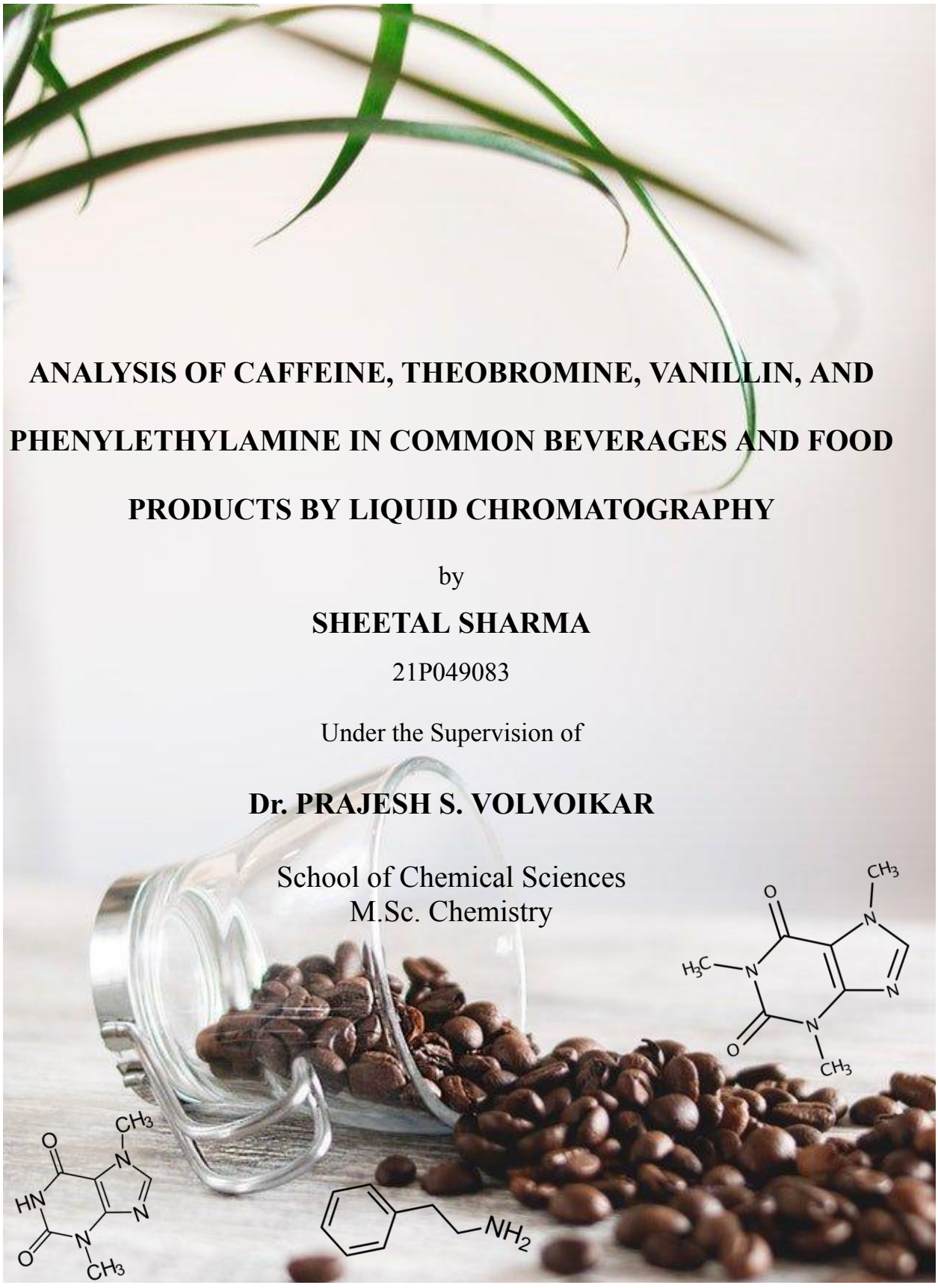

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The completion of this dissertation project, “Analysis of Caffeine, Theobromine, Vanillin and Phenylethylamine in Common Beverages and Food Products by Liquid Chromatography” would not have been realistic without my dissertation guide, Dr. Prajesh S. Volvoikar, who's advice, patience, support, and feedback was vital throughout the study process. He has my sincere gratitude. This dissertation's ultimate form was shaped by his knowledge and commitment to my fulfilment.

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INTRODUCTION

Liquid chromatography (LC) is an analytical separation technique for identifying and estimating numerous chemical compounds in food and drink samples. Scientists and researchers acquire significant insights into the nutrient content, potential health benefits or hazards, and identify potential contaminants or illicit ingredients by analysing the chemical makeup of foods and beverages. This technique includes sample preparation, chromatographic separation with the appropriate method development, detection and estimation of the analytes. Depending on the kind of application and analysis demands, this methodology can be performed using several types of liquid chromatography, such as high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography (UHPLC), ion chromatography (IC), etc.

In many cultures worldwide, tea, coffee, chocolate, and cocoa are all cherished libations and delicacies. Some of their cultural relevance and value are highlighted below:

1. Tea- One of the most popular drinks in the world, tea has a long cultural history in India, Japan, China and the United Kingdom, where it is sipped throughout the workday and frequently offered to visitors as a sign of hospitality. Tea preparation and presentation are frequently viewed as gestures of respect.

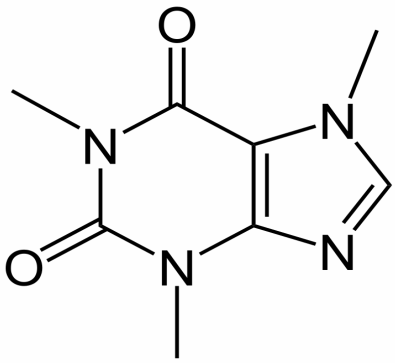
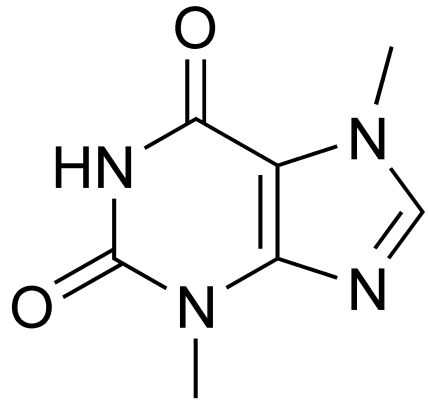
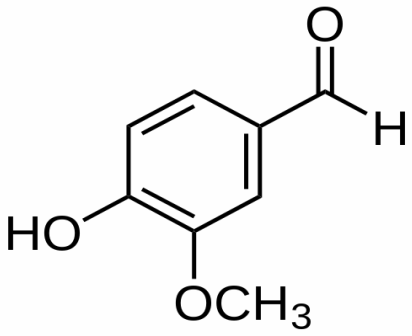
2. Coffee- Another popular beverage with cultural importance is coffee, which is regarded as a fuel for social interaction. From cold brew to drip coffee to espresso, it is enjoyed in various ways. With specific directions for preparation and presentation, coffee culture is a way of life in nations like Italy and France. Coffee rites are still an integral part of daily life in other parts of the world, such as Ethiopia and Yemen.

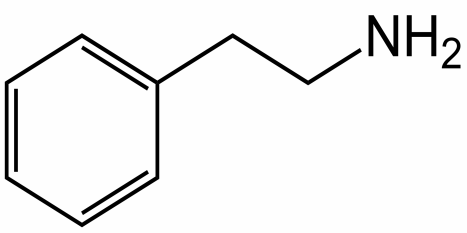
3. Chocolate- The first written mention of chocolate was as a bitter beverage in the early human civilizations of Central America. Present-day chocolate consumption spans the globe and is a renowned sweet delicacy. Chocolate appears to be an art form with exquisite shapes and flavours in nations like Belgium and Switzerland. Chocolate continues to be included in traditional dishes and is a significant component of regional cuisine in other parts worldwide, in places like Mexico and Central America. The chemicals found in chocolate induce a reaction in the human brain that changes how we feel. Chocolate contains bioactive substances like polyphenols, carbs, proteins, and lipids.

4. Cocoa- the primary ingredient in chocolate, is derived from beans that grow on Theobroma cacao, generally called the cacao tree. The tree is indigenous to South and Central America's tropical regions, although it is also grown in West Africa and Southeast Asia. In South America, cocoa is a key ingredient in regional dishes and is often used in traditional recipes. As a sacred plant, cocoa was employed in religious rituals in several cultures, such as the Aztecs. The health advantages of cocoa have recently been recognized, and it is a common addition to protein bars, smoothies, and other healthy foods.

Products from plants like tea, coffee, chocolate, and cocoa all contain chemicals like Caffeine, Theobromine, Flavanoids, Tannins, Polyphenols, Theophylline, and Melatonin. These substances help give tea, coffee, chocolate, and cocoa their distinct flavours, aromas, and health advantages.

Some prominent chemicals found in chocolates are-

Caffeine [2]		(1,3,7-trimethyl xanthine) Pharmacologically active substance. Odourless white crystalline powder, bitter in taste. Mild central nervous system (CNS) stimulant. The recommended daily dose for the pharmacological effect is 200mg/day.
Theobromine [3]		A dimethylxanthine is classified as a Xanthine alkaloid. Pharmacologically active substance. White crystalline powder, bitter in taste. Derived from theobroma (contains no bromine). A natural cough medicine. Smooth muscle relaxant. Used as a vasodilator. Improves the state of alertness in humans. Strengthens tooth enamel. The recommended daily dose is 250 to 500 mg daily.
Vanillin [4]		A phenolic aldehyde that acts as a flavoring agent made from vanilla. It imparts creaminess to the chocolate while balancing the cacao's bitter flavor. Many vital medicines are manufactured using it as a chemical intermediary.

PEA [5] **Phenylethylamine**		An organic alkaloid connected to amphetamines, also known as a love drug. Great attentiveness and a contented feeling are brought on. In the human neurological system, it acts as a neurotransmitter.

Since such complex chemicals are constituents of these delicacies, it is very important to keep in check their quantity used. More than the recommended quantity of any chemical is harmful and can bring about negative effects on the human body.

Nowadays, humans consume more than the required amount of caffeine, intentionally or unintentionally, in the form of tea, chocolates, coffee, cold drinks, etc. The high amount of caffeine (and other constituent chemicals) in the body leads to increasing dependency on such chemicals, anxiety, irritability, nervousness, heart palpitation, headaches, and excess release of acid in the stomach. It can also lead to premenstrual symptoms in women who overuse it.

Hence, it is important to be aware of the serious effects of caffeine and other chemicals in popular beverages when it's over-consumed in any form. Also, it is very important to have knowledge about daily recommended doses of these chemicals for pharmacological effects. One of the most important areas of research in food and beverages is liquid chromatography analysis. This is because various chemical substances like sugars, organic acids, flavourings, preservatives, or pollutants are present in food and beverages. These chemicals must be measured accurately for safety, regulatory compliance and quality control.

HPLC analysis is used in most analytical labs. If the the stationary phase is silica with polar functionality, then it is classifas thed as normal phase. If the stationary phase is modified non polar functionality is referred to as reverse phase. Their time on the column is determined by the specific intermolecular interactions of the solutes (polarity) with the stationary phase. As the sample's solutes pass through the column, they separate from one another in a process known as differential migration. In the chromatogram, each peak reveals the sample element in relation to a standard for that peak. The peak's area corresponds to the quantity. A solvent reservoir, a pump, an injection valve, a column, a detector, and a data processing unit are the typical components of an HPLC system (Figure 1). A pulse-free and continuous flow from the pump is essential in order to minimize the drift and noise of the detector signal. The term "LC-MS" refers to the method that combines MS and HPLC, wherein a mixture's distinct components are first separated, then the components are ionized and separated according to their m/z ratio. Combining the two analytical techniques increases accuracy and decreases experimental error. [6]

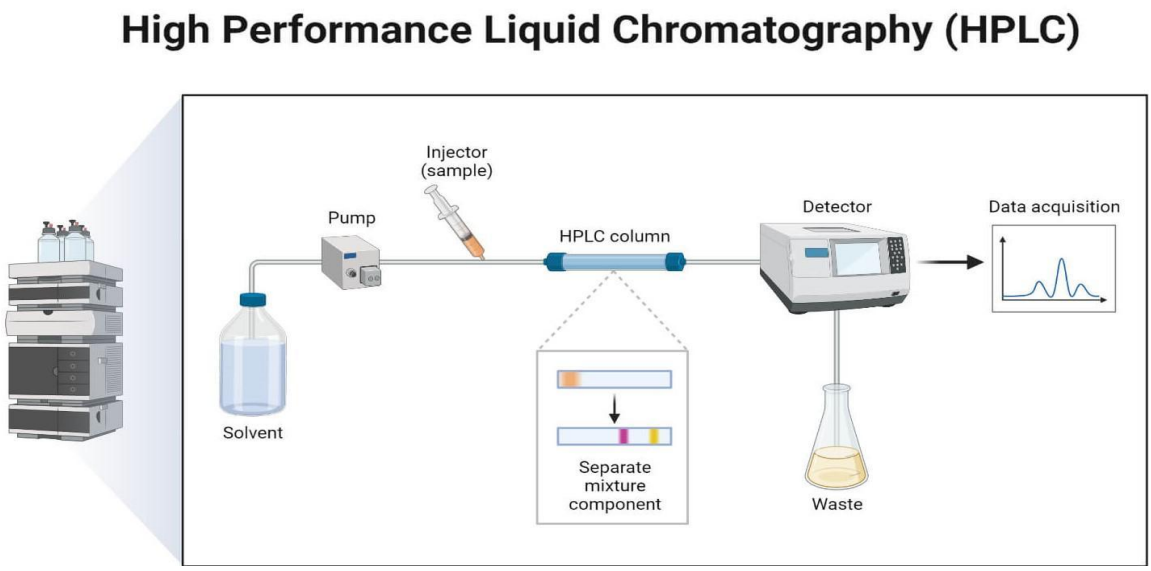


***Figure 1**: HPLC instrument* [7]

LITERATURE REVIEW ON RESEARCH PAPERS

The literature data used during this dissertation topic is listed below. Basic highlights of the most important theories, research findings, and methodological approaches, as well as highlighting the gaps and restrictions in the existing literature.

Kreiser *et al* [8] in 1978 analysed caffeine and theobromine in cocoa and chocolate products using two methods for analysis. Chocolate sample for analysis were grated and passed through 8 mesh screens to remove larger particles and bring uniformity.

In first method for extracting fat from chocolate bars was done using petroleum ether in two shaking steps and centrifugation at 200 RPM for 10 minutes. The solvent was decanted and then freed of solvent by placing the test tubes in a warm water bath. After drying, the sample was weighed and quantitatively transferred with HPLC water to a flask containing boiling chips. Spiking was done for recovery purposes by adding known amounts of Theobromine or Caffeine. As a result, the Spike almost doubled the number of alkaloids in the original sample. HPLC water was added to make it up to 95 ml, and it was heated at 100°C for about 25 minutes. After cooling to 20°C, HPLC water was added to the flask, which was then stopped and carefully shaken. The contents were then poured into the centrifuge tube and centrifugation was done at 200 RPM for 5 minutes. The supernatant was filtered and the sample was injected into the system. Filtered standard solutions were also injected. The chromatogram also showed identifiable peaks of theobromine and caffeine with additional peaks. It was observed that not all amounts of theobromine could be extracted from the sample because of its relative insolubility in water. Also, the residue of theobromine was found to be

contaminated during the filtration. Extraction of theobromine from tetrachloroethane solvent was not quantitative as well. Because of the Limited versatility of this method, the analysis could not be done on all types of chocolate samples. The second method HPLC method was then employed, where the column was of C18 reverse phase 10 µm particle size. The mobile phase was Methanol-water-acetic acid in the ratio of 20:79:1 respectively. Extraction of the sample was done by simple hot water extraction for 25 minutes using the defatting solvent. A very good amount of alkaloids was added to the measured volumes of the solvent and left for three days with occasional shaking. Each solution was filtered and the solvent was evaporated. Residue obtained was dissolved in water and diluted to a suitable volume. These solutions were then injected into the HPLC system. The standard solutions were re-purified, filtered, and stored. Quantification was done at 280 nm. The two analysis methods were then compared and it was concluded that the HPLC method could be done within 2 hours after the sample was weighed while the official method required almost 1.5 days. The HPLC method did not require the use of chlorinated solvents while the official method did.

Thomas *et al* [9] used reverse-phase liquid chromatography to simultaneously eestimate caffeine, theophylline and theobromine. The mobile phase used for this method was acetonitrile: water in the ratio of 10:90 respectively, pH was adjusted to 2.5 using acetic acid and the flow rate was maintained at 1.5 mL/min. Quantification was done at 274 nm and the analysis time was observed in less than 15 minutes. From each chocolate bar, 1g of sample was taken in a centrifuge tube and melted in a water bath at 50 °C for 15 min. To the melted chocolate,1ml of internal standard (β-Hydroxyethyl theophylline) was immediately added and agitated in an ultra-sonicator bath (approx. for 5min) until there was a homogeneous mixture formed. To eliminate the matrix in cocoa-based

samples, Soxhlet and solid-phase extraction techniques were used. Samples were defatted with hexane solvent (4 times extractions). The mixture was agitated (using an ultrasonicator bath) for 20 min and then centrifuged for 10 min. The hexane layer, which contained the lipids, was then removed. Nitrogen gas was used to remove the hexane instead of placing samples in a warm water bath to remove the solvent, therefore, eliminating the need to quantitatively weigh the dried sample before transferring it into a sample vial for LC analysis. HPLC water (30 mL) was added to the dried chocolate and agitated in an ultrasonicator bath for approximately 15 min to ensure dissolution of the sample prior to filtering. The sample was centrifuged and the supernatant was filtered and an aliquot of the filtered sample was transferred into a sample vial for LC analysis.

HPLC grade water was used to prepare the calibration standards for caffeine, theobromine, theophylline, and β-Hydroxyethyl theophylline was added as an internal standard-1000 µg/mL. Each solution was injected in HPLC before sample analysis to get the detector response and retention time factors. The chromatogram showed the separation of standard mixtures in the order of theobromine, theophylline, β-Hydroxyethyl theophylline, and caffeine. All separations were conducted at room temperature (~25 °C). It was observed that the samples were homogenous and free from matrix interferences. Simultaneous separation of the analytes in the SRM 2384 Baking Chocolate was observed.

Siva Prasad and co-workers [10] used high-performance thin-layer chromatography (HPTLC) to estimate caffeine content in chocolates. The mobile phase used was Butanol- chloroform- ammonia- acetone in 4:3:2:1(v/v/v/v) respectively. Measurement was done by densitometry at 254 nm.

Cocoa powder (500 g) was weighed and dissolved in 1000 mL hot water with proper stirring. From this solution, 250 mL was added in a 1000 mL separating funnel.

Then 175 mL of dichloromethane and 175 mL of chloroform were added to the separating funnel. The separating funnel was then shaken well and allowed to settle for 1.5 hours without disturbance. Then, the organic layer was collected from the separating funnel into a beaker and then again 25 mL of dichloromethane and 25 mL of chloroform were added to the separating funnel and allowed to settle for 1 hour, again the organic layer was collected and combined. Sodium sulphate was added to the organic solution collected in the beaker and stirred well till it became free flowing and then the solution was filtered and kept for evaporation at room temperature (at room temperature, dichloromethane and chloroform get evaporated) hence the precipitate was obtained after evaporation. After method development, the optimisation of standard caffeine and extracted caffeine from the cocoa powder was done. Spots were clearly visible and the method was continued for validation and was found to be accurate, linear, and precise.

Henderson and co-worker [11] separated xanthines using rapid resolution HPLC. Reversed-phase HPLC having a C18 column and an isocratic method was employed. It was seen that time was reduced for analysis from 8 minutes to 1.5 minutes without major loss in resolution could be done by reducing the column length and particle size (from 4.6 × 250 mm, 5 µm to 4.6 × 50 mm, 1.8 µm). Ion pair chromatography has also been mentioned for xanthine separation. First, the selection of the stationary phase was made to get the most suitable one which could give the best overall separation with the shortest time for analysis. ZORBAX Stable Bond phase C18 became the stationary phase of choice. The mobile phase used was A= 0.2% Formic acid (FA) in water and

B=Acetonitrile with 0.2% FA, at isocratic composition at 98% A and 2% B (v/v). The flow rate adjusted was 1.5 mL /min. Change in the column configuration to increase the speed while maintaining the resolution was done by changing the analysis time from 8 mins to 1.5 mins. Three liquid samples *viz* chocolate syrup, hot cocoa, and black tea (bag) were prepared using the directions mentioned on the container. After preparation, all solutions were centrifuged, and then the aqueous layer was filtered and injected into the HPLC column. From this paper, it is seen that by reducing column length and particle size to a certain extent, the overall efficiency and resolution can remain nearly the same which gives a shorter time analysis. Since the flow rate was 1.5 mL/min the solvent used decreased consequently resulting in a decrease in the overall cost. Also, since the column was made shorter the peaks were seen as narrower which also led to less use of sample volume. But one of the main disadvantages to be taken care of is when the particle size reduces there is a probability of column back pressure increasing which can result in poor analysis and resolution and can also make the column unstable.

Pérez-Esteve et al [12] estimated vanillin and ethyl vanillin in cocoa powders by HPLC. Simple extraction of vanillin and ethyl vanillin is done in cocoa powder followed by quantification using HPLC with analysis time of around 4 minutes.

1 g of cocoa powder was weighed and suspended in 10 mL of methanol: water (1:1, v/v), for 5 minutes the mixture was continuously shaken and then passed through a 0.45 µm pore-size nylon filter. The solution was quickly injected into the system or stored in a laboratory freezer. The column used in HPLC was the C18 column (RP-HPLC) and the mobile phase used was MeOH and water. The process started with a linear gradient from 50% to 100% MeOH for 2 min, followed by an isocratic elution with 100% MeOH

for 2 more min. The HPLC system was again equilibrated with the initial composition for 5 min prior to the next injection. UV detection was done at 231 nm. In all cases,10 µL was injected and the flow rate was adjusted to 1.2 mL/min as it was observed when the flow rate increased from 1 mL/min to 1.2 mL/min there was a decrease in the retention time and the peak became narrower. Efficient and reproducible separation was observed and separation was obtained in less than 4 min, which allows a quick quantification of cocoa sample flavoring with both vanillin and ethyl vanillin flavors.

Bispo *et al* [13] estimated caffeine, theobromine and theophylline concurrent determination by HPLC. Beverages and urine samples were analysed using the Reverse Phase-HPLC method having Bondesil C18 column. Mobile phase: methanol–water–acetic acid or ethanol–water–acetic acid (20:75:5, v/v/v) with UV detection at 273 nm. The flow rate was set to 0.7 mL/min. Selection of mobile phase was done by analysing the chromatograms for respective standards: (A) mobile phase MeOH-Water (40:60, v/v) (pH = 6.14) and (B) MeOH–water–acetic acid (20:79:1, v/v/v) (pH = 3.12). It was observed (A) Showed poor resolution and overlapping of peaks whereas (B) showed better peak resolution as compared. Preparation of methylxanthine calibration solutions was done by diluting methylxanthine mixture stock solutions with water within the concentration range of 0.25 to 60 µg/mL and stored in the dark-glass flasks at 4°C. The sample was prepared with 0.2 g chocolate per 20 mL water and was cleaned from grease by Soxhlet extraction with hexane solvent. 20 µL sample injection was made. The elution was in the order of theobromine, theophylline, and caffeine. When the mobile phase methanol–water–acetic acid (20:75:5, v/v/v) analysis was done within 12 minutes, and when the mobile phase ethanol–water– acetic acid (20:75:5, v/v/v) was used, the analysis time reduced to 6 minutes. It was observed that most of

the methods described in the literature for methylxanthine analysis use mobile phases containing only methanol–water which allows caffeine quantitation but does not show good resolution of theophylline from theobromine. Methylxanthines may suffer protonation, which results in ionic species stabilized by resonance and inductive effects. If the pH decreases below 4, xanthines become protonated and the interaction with C18 reverse-phase columns increases hence increasing the retention time and poor resolution. To counter this problem acetic acid was added to the mobile phase to increase the acidity of the system which resulted in good separation. The stability of the methylxanthine standard played an important role in getting good separation results. The standards were prepared in methanol or ethanol and stored in dark glass flasks at 4°C. They showed good stability.

Hurst and co-workers' [14] estimated four biogenic amines in chocolate using HPLC. To analyse and quantify 4 biogenic amines presumed to occur in chocolate tyramine, tryptamine, 2-phenylethylamine, and serotonin were chosen as the amines of interest. Analysis was done using two RP-HPLC system UV detection at 254 nm using Bondapak column C-18. The HPLC systems had different flow rates and fluorescence detectors of different wavelength filters. Mobile phases used were also different operating at different pH. Samples were first defatted with petroleum spirit. 1 gram of defatted chocolate was mixed with 20 mL of 0.1 N perchloric acid using an omni-mixer setting for 10 min. The mixture was then transferred to the centrifuge tube for centrifugation at 2000 RPM for 10 mins. A concentrated ammonia solution was added to the supernatant liquid to adjust it to pH 10.3 and stored overnight in a refrigerator at -4℃. The solution was then filtered using Whatman No. 41 filter paper. Solid sodium chloride was added to the filtrate to make it saturated and then extracted four times with

5 mL ethyl acetate - acetone (2: 1) mixture. After each extraction, the mixture was centrifuged to help separate the two layers faster. The first three extractions were transferred into a clean test tube while the fourth extraction was filtered through Whatman phase-separating paper and then transferred to the test tube. The water-free extracts were evaporated to dryness under nitrogen at 20°C and then dissolved in 1mL of the HPLC mobile phase. 20 µL of the extract was injected into the HPLC system and compared with injections of standards. The two HPLC systems allowed the direct measurement of the amines by natural fluorescence phenomenon. The concentration of the amine in the extract was calculated by comparing the peak heights of the sample and the standards. From the chromatogram, it was seen that phenylethylamine got eluted as the third component within 10 mins of analysis and had the highest intensity as compared to the other three amines of interest. It was observed that the levels of the amines of interest were quite low in chocolate as compared with the levels found in the other products. Hence, this method may not be a comprehensive study of the amines that occur in chocolate and more explorations need to be made.

Koehler and co-workers [15] used HPLC for analysis of phenylethylamine, tyramine & tryptamine in sausage, chocolate, and cheese. For analysing biogenic amines in food products, paired-ion chromatography and HPLC provided a quick, efficient approach with UV detection at 254 nm.

10 g of the sample was mixed with 100 mL of 0.1 N perchloric acid using an omni-mixer setting to 15 min to homogenize and then centrifuged for 20 min at 10,000 x G at 4°C and the fat layer was removed. The supernatant was adjusted to pH 7.0 by adding ≅1.5 g of KOH and left overnight at 4°C to allow the formation of a precipitate. It was then filtered and degassed under vacuum for 15 min, adjusted to pH 6.5 with 1N HCl, and

registered to a weak cation exchange column to purify and concentrate the extracts (pH adjusted 6.5; distilled water was used to wash the column; the adsorbed bases were then eluted with 1N HCl). Analysis was done using HPLC and the chromatogram was obtained. Prior to the injection in the HPLC, the extract obtained from the cation exchange column was first adjusted to pH 6.5 and was diluted using distilled water to make it up to 35 mL. 20 µL of the extract was then injected into the HPLC column. The flow rate was adjusted to 1.2 mL/min. The mobile phase used was methanol-water (35:65) containing 0.005M 1-heptane sulfonic acid as a counter-ion. Mixtures of tyramine, phenylethylamine, and tryptamine were separated by HPLC using a nonpolar octadecylsilane-coated solid support (reversed-phase) with heptane-sulfonic acid counterion in the solvent. The effectiveness of the separation was estimated by doing a comparative study by changing the mobile phase composition and the flow rate. The mobile phase ratio of methanol: water (35:65) gave the best resolution as compared to others. It was observed that by increasing the flow rate to 1.2 mL/min, the loss in resolution did not occur and it remained almost the same, however, it gave a shorter analysis time from 26 min to just 15 min. Hence the flow rate of 1.2 mL/min was selected. None of the chocolate samples showed any detectable levels of tyramine, phenylamine, or tryptamine.

Risner and co-workers [16] used HPLC for determination of flavour enhancers in chocolate products and artificial flavors. A methanol–water gradient mobile phase was used to elute the compounds of interest using a reverse-phase C-18 column. UV detection using a wavelength of 273 nm was used to monitor the eluent. Chocolate sample extracts were heated to 60 ± 2 ◦C for 10 min in a water bath to melt the sample. After extraction, the extract was then filtered (0.45 µm polyvinylidene fluoride) into a vial.

The injection volume was 5 µL into the HPLC column. The mobile phase flow rate was 500 µL/min using a gradient composed of (A) 0.3% acetic acid in water and (B) MeOH. A concentration gradient was used from A being 85% (10 mins) to 75% (8 mins) to 70% (7 mins), followed by column wash of 100% methanol for 5 mins and conditioning of column for 5 mins using 100% A, before next injection. A lot of analysis was done to choose the perfect mobile phase and the flow rate for good resolution of the peaks. This method was capable of simultaneously quantifying theobromine, catechin, vanillic acid, caffeine, vanillin, epicatechin, and ethyl vanillin from the water extract of consumer products. The sample preparation required was minimum and the analysis can be done at convenience as equipment required for this method is present in most laboratories. It is the first reported single procedure to determine theobromine, catechin, caffeine, and epicatechin simultaneously in Standard Reference Material 2380 baking chocolate without large-scale sample preparation.

Vries *et al.* [17] analysed caffeine and theobromine content of various types of cocoas using HPLC. Mobile phase used was: Water-methanol-acetic acid (90:10:1), with UV absorbance at 280 nm. Samples were ground to pass through 200 mesh sieves. 1 g of sample was transferred to the flask and approximately 100 mL of hot water was added. The mixture was stirred for 30 minutes by putting a magnetic stirrer bar in the flask. After this the sample was allowed to cool down and the magnetic stirrer bar was removed by washing it with water in the flask itself. The sample was then centrifuged and filtered and the clarified aqueous solutions were transferred to the vials for injection into the HPLC system. A flow rate of 1.5 mL/min was maintained and the system was equilibrated for 1 hour. 20 µL of standard solution and sample solution was injected accordingly. The concentration of caffeine and theobromine was calculated by

comparing the peak height and area with the standard and the sample peaks from the chromatogram obtained. HPLC quantification was based on the method of Kreiser and Martin (1978) with the exception that the hexane defatting step was removed.

Pokhrel et al. [18] analysed caffeine content in coffee & tea using HPLC. Column used for analysis was C-18 using mobile phase as methanol: water- 40:60. Retention time of caffeine in sample extracts observed was 2.66 mins at 275 nm UV detection. First, the tea and coffee samples were ground into a fine powder, then measured out into 250 mL conical flasks, weighing around 0.3 g each. The mixture was then placed over a water bath with 200 mL of distilled water added. A half-hour was spent on extraction. The solution was then cooled, its volume kept at 250 mL and it was filtered. Filtrate (1 mL) was pipetted into a clean 10 mL volumetric flask and made up to the mark with HPLC water. Filters were used to fill HPLC vials with the prepared sample for examined.

CONCLUSIONS FROM LITERATURE REVIEW:

While doing the literature review, it was observed that few analytical methods had been reported for the simultaneous determination of caffeine, theobromine, theophylline, vanillin, and phenylethylamine in various food products. Also, some of these techniques call for laborious sample preparation, while others demand heavy loads of inorganic components, which could interfere with HPLC analysis and are unsuitable for the simultaneous separation of caffeine from interfering metabolites.

Other methods may not provide accurate and precise results (higher standard deviation) as the resolution between the peaks may not be good enough for quantification.

Some methods required the re-purification of standard solutions almost every two weeks and storage at very low-temperature conditions. Only a few methods have been applied for the analysis of food products for the determination of all compounds of interest in the same sample.

Most methods reported have used Reversed-phase high-performance liquid chromatography (RP-HPLC), more specifically RP-HPLC coupled with mass spectrometry or HPLC with ion-paired chromatography, etc. The precision of this method was shown to be good for the selective determination of xanthine compounds over the concentration range of 5 µg/mL to 40 µg/mL.

Through the development of the technology to pack columns efficiently with very small particle supports and prevent the possibility of back-pressure being formed, which causes the column to become unstable and ultimately destroys the packing of the column, HPLC has achieved significant advancements. HPLC is a quicker, simpler process that also facilitates sample quantification. It is clear that HPLC is useful for quantifying practically all food products with cocoa solids (HPLC is usually coupled with

other instruments). Mostly the separation time is short and precise with accurate results. No chemical reactions are necessary to form volatile derivatives for HPLC analysis while it may be necessary for gas chromatography.

The literature review revealed that methanol is one of the primary solvents utilized in liquid chromatography. Its usage in place of ethanol in the mobile phases of the HPLC system results in a reduced cost and nearly identical separation of xanthines (caffeine, theobromine and theophylline).

For the analysis of phenylethylamine, a few points were concluded: Since amines are basic and ionizable, HPLC in conjunction with ion-pair chromatography is feasible for biogenic amine separations. The association of the positively charged counter-ion (eg. heptane sulfonic acid) results in a lipophilic ion-pair that can be separated by nonpolar (reversed phase) chromatography.

Results were obtained by NIST regarding theobromine, caffeine, and theophylline where the samples were defatted with hexane four times, dried in a stream of nitrogen gas and restored in water. The extract was analysed using UV-absorbance detection at 274 nm and quantified using an internal standard (β-hydroxyethyl theophylline). The isocratic mobile phase of acetonitrile-water-acetic acid was used.

Whereas in another research paper, the procedure described was similar to the mentioned above except that hexane was not used and the results were calculated based on the external standards. The mobile phase gradient used was methanol-water-acetic acid.

While reading the research papers, many significant points were noted, including the necessity of performing numerous analyses to select the best mobile phase for the high-resolution separation (instead of just applying the mobile phase recommended in

the literature) in order to ensure accurate and precise quantification. To determine the optimal solvent system for the separation, the percentage composition of the same mobile phase was altered to varying degrees, and the chromatogram of each run was compared.

In addition to the mobile phase, the system's flow rate was a crucial element in deciding on the shorter analytical time. It was found that a certain amount of flow rate increase was possible with a reduction in column particle size without sacrificing peak resolution, which resulted in a significant reduction in analysis time.

For the estimation of vanillin, it was noted that any method devised for vanillin extraction must be able to cope with impurities inherent in a fatty solvent, especially those which lead to the formation of emulsions as the extraction of vanillin from the samples, lots of different reagents were used. Even slight excess of any of them caused the impurities to be extracted along with crude vanillin during subsequent extractions.

It was noted that during the estimation of caffeine and theobromine from the aqueous extract of the chocolate samples, the interfering cocoa pigments could be removed effectively by passing through Sep-pak C-18 cartridge (avoiding the possibility of reduction in the efficiency of the column and shortening of its life which is caused by aqueous extracts when directly injected on analytical column) before going to the main analysis column using acetonitrile: water (20:80) as mobile phase. Hence, the resolution of the peaks and the accuracy were well maintained.

AIM AND OBJECTIVES

"**Analysis of Caffeine, Theobromine, Vanillin and Phenylethylamine in Common Beverages and Food Products by Liquid Chromatography**"

It's crucial to comprehend how a few milligrams of these compounds can have a significant impact on a person's conduct and thought processes. Therefore, viewing this from the perspective of analytical chemistry encourages estimating the number of relevant compounds and becoming familiar with the HPLC system and its handling, which is a key technique for the separation of solutes in a sample, based on differential migration of solutes on a column under high pressure along with mobile phase solvent system.

Objectives:

- To develop a reliable liquid chromatography method for separating and estimating caffeine, theobromine, vanillin and phenylethylamine in selected food and beverage samples.
- To practically comprehend the parameters for method validation and standard stability.
- To conduct spiking studies to identify target analytes in complex sample matrices.
- To gain practical experience with the HPLC, LC-MS system and UV-Vis spectrophotometer.

EXPERIMENTAL WORK

TThe analysis of caffeine, theobromine, vanillin and phenylethylamine by liquid chromatography started with carefully selecting samples. The samples included milk chocolate, cocoa powder, coffee beans, cocoa beans, and green tea (tea bags).

Each target chemical's standards were ordered and obtained. Sisco Research Laboratories Pvt. Ltd. (SRL) provided three reference materials: caffeine anhydrous (pure 98%), vanillin (pure 99%), and 2-phenylethylamine (pure 99%), while Tokyo Chemical Industry (TCI) provided the fourth reference materials: theobromine (pure >98%). Standards were not procured as they were expensive.

Before beginning sample preparations, it is critical to investigate the chosen standards' stability. Standard stability testing is crucial to analytical chemistry that ensures accurate and dependable analytical results. The following were the general stages of doing a stability test:

- The standard solution was prepared by diluting 1.0204 g of caffeine (according to the 98% assay mentioned on the bottle) in distilled water to make a 1000 ppm stock solution in a 1000mL volumetric standard flask.
- 10mL was pipetted out, transferred to a 100mL volumetric flask, and diluted with distilled water to the mark. As a result, 100ppm of the standard solution was prepared.
- From this 100ml standard solution, a series of concentrations was prepared- 5ppm, 10ppm, 15ppm, 20ppm, and 25ppm by pipetting out 5, 10, 15, 20, and 25 mL respectively in 100mL standard volumetric flasks. These were labeled properly.

- The standard solutions were evaluated using an Ultraviolet-Visible spectrophotometer (absorbance was determined) at defined time intervals (the first day, the seventh day, and after a month). Any changes in the absorbance of these standards were monitored and observed throughout the course.
- The data was analyzed by graphing the standard's absorbance vs a series of standard concentrations to determine the standard's stability over time.

Similarly, the other three standards, vanillin, 2-phenylethylamine (in distilled water), and theobromine (in methanol using a sonicator), were prepared and tested for standard stability. The stability test also assisted in determining the maximum wavelength (λ_{max}) for subsequent liquid chromatography analysis (Figure 2).

Figure 2: Series of standards** and **Shimadzu Uv-Vis spectrophotometer

After the initialization of the Uv-Vis spectrophotometer, Spectrum mode was chosen for maximum wavelength determination. The record range was set from 0 to 2.5A and the scan range was set to 800 nm~200 nm. Both the quartz cuvettes (filled with distilled water) were placed in respective reference and sample compartments. Baseline correction was done by clicking F_1 and waiting till the beep sound came. The sample compartment cuvette was removed and rinsed with the 20ppm standard solution before being replaced in the sample compartment. The Start button was pressed, then F_2, and

finally, 3 was pressed. The maximum wavelength was measured. The return button was used to return to quantitative mode, and number 1 was pressed to set the maximum wavelength value. The absorbance was then measured directly, beginning with the lowest concentration solution.

The λ_{max} for,

- The caffeine standard obtained was 271nm.
- The Theobromine standard obtained was 272 nm.
- The Vanillin standard obtained was 282 nm.
- The 2-Phenylethylamine standard obtained was 257nm.

Caffeine and Theobromine were shown to be stable following analysis on the first, seventh and 30th days. However, Vanillin and 2-Phenylethylamine exhibited less than a week's stability. That is, for any analysis, Vanillin and 2-Phenylethylamine standard solutions have to be prepared fresh and analysed within 2-3 days.

Following are the graphs plotted-

[A] CAFFEINE

DAY-1

S No.	Concentration (ppm)	Absorbance(at 271nm)
1	5	0.53
2	10	0.73
3	15	1.1
4	20	1.42
5	25	2.18

(Table 1.1 Caffeine standard)

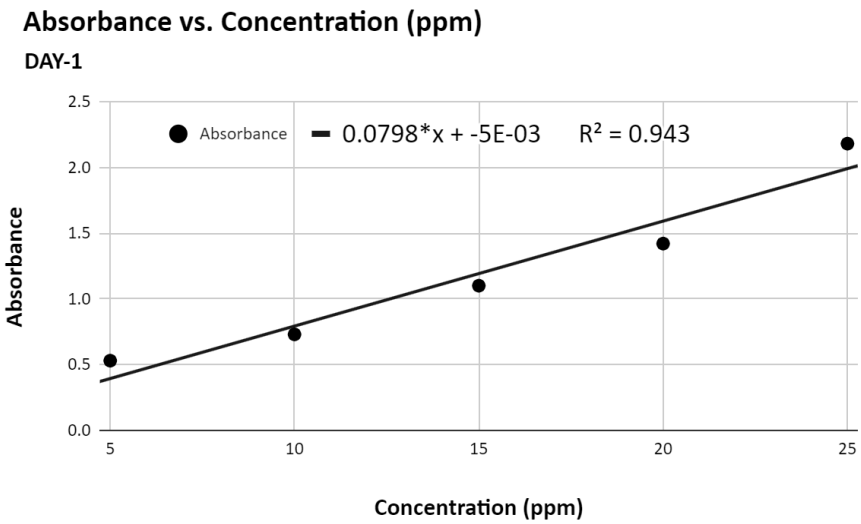


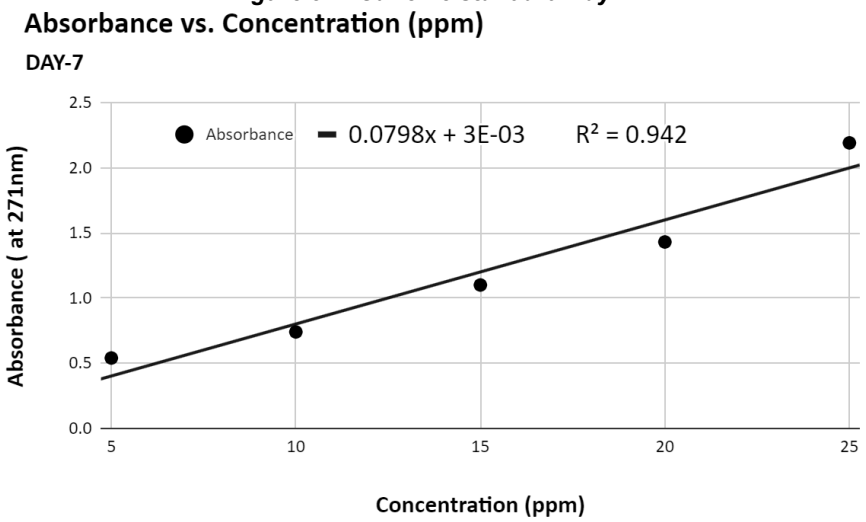
Figure 3.1: Caffeine standard Day-1

DAY-7

S No.	Concentration (ppm)	Absorbance (at 271nm)
1	5	0.54
2	10	0.74
3	15	1.1
4	20	1.43
5	25	2.19

(Table 1.2 Caffeine standard)

Figure 3.2: Caffeine standard Day-7



DAY-30

S No.	Concentration (ppm)	Absorbance (at 271nm)
1	5	0.55
2	10	0.74
3	15	1.12
4	20	1.44
5	25	2.16

(Table 1.3 Caffeine standard)

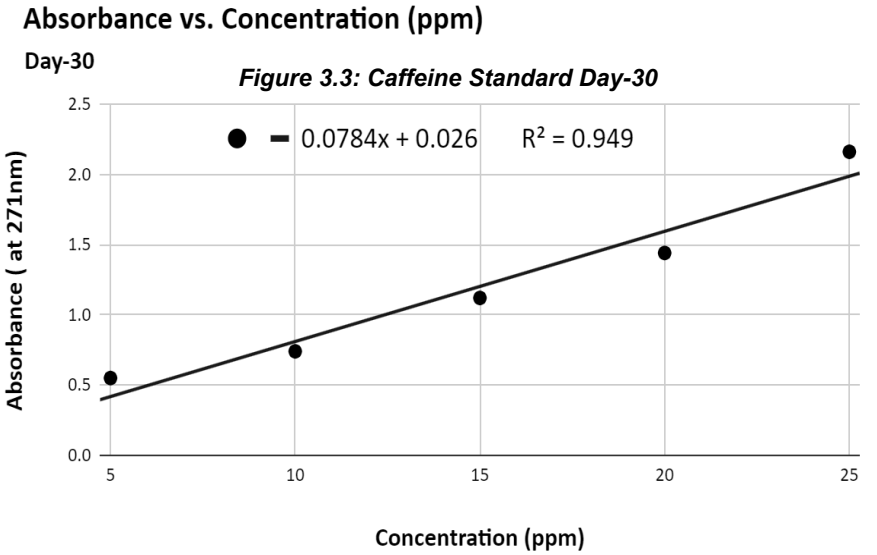


Figure 3.3: Caffeine Standard Day-30

[B] <u>THEOBROMINE</u>

DAY-1

S No.	Concentration (ppm)	Absorbance (at 272 nm)
1	10	0.529
2	15	0.792
3	20	1.05
4	30	1.56

(Table 1.4 Theobromine standard)

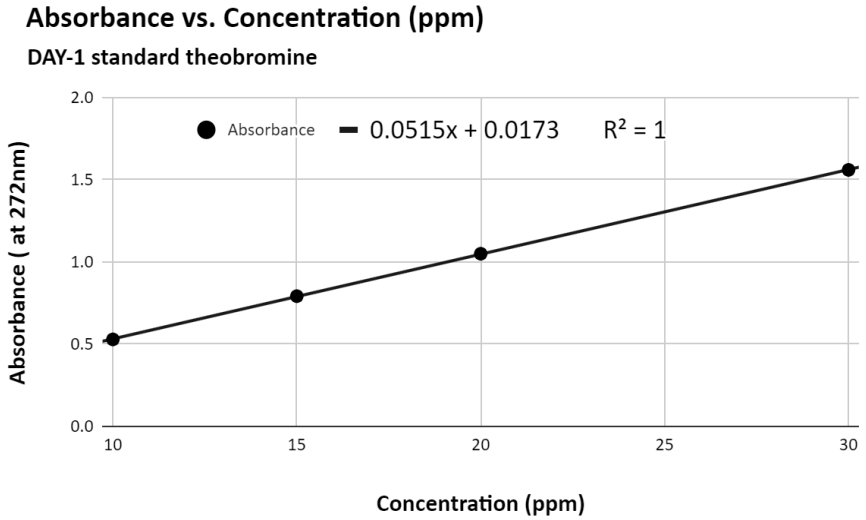


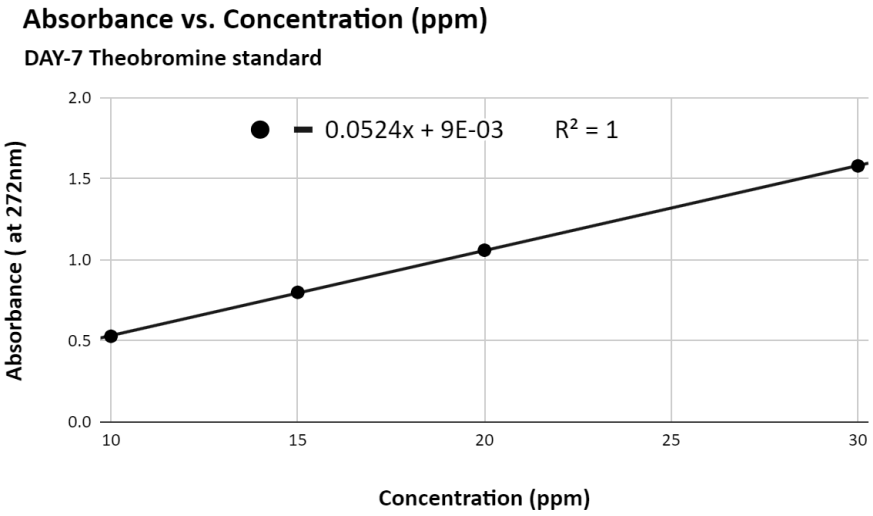
Figure 3.4: Theobromine standard Day-1

DAY-7

S No.	Concentration (ppm)	Absorbance (at 272 nm)
1	10	0.529
2	15	0.8
3	20	1.06
4	30	1.58

(Table 1.5 Theobromine standard)

Figure 3.5: Theobromine standard Day-7

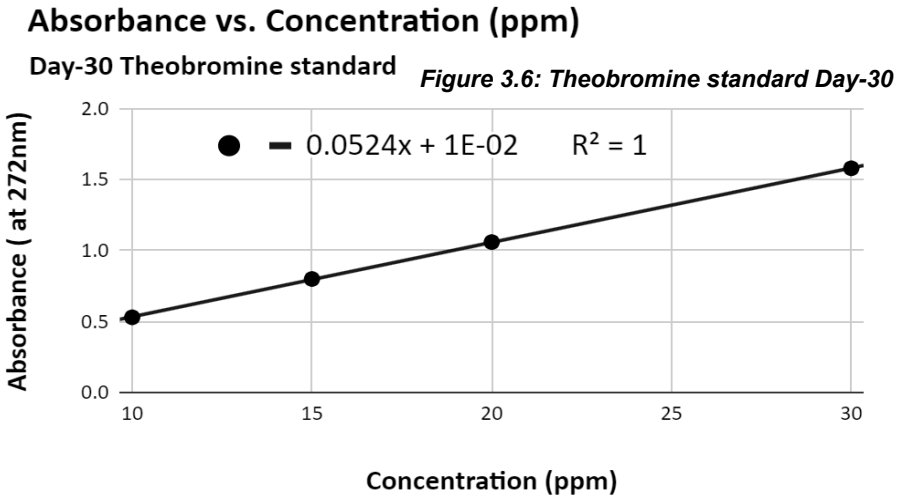


DAY-30

S No.	Concentration (ppm)	Absorbance (at 272nm)
1	10	0.53
2	15	0.8
3	20	1.06
4	30	1.58

(Table 1.6 Theobromine standard)

Figure 3.6: Theobromine standard Day-30



C] <u>VANILLIN</u>

DAY-1

S No.	Concentration (ppm)	Absorbance (at 282nm)
1	20	1.02
2	25	1.47
3	30	2.00
4	40	2.54
5	50	3.61

(Table 1.7 Vanillin standard)

Figure 3.7: Vanillin standard Day-1

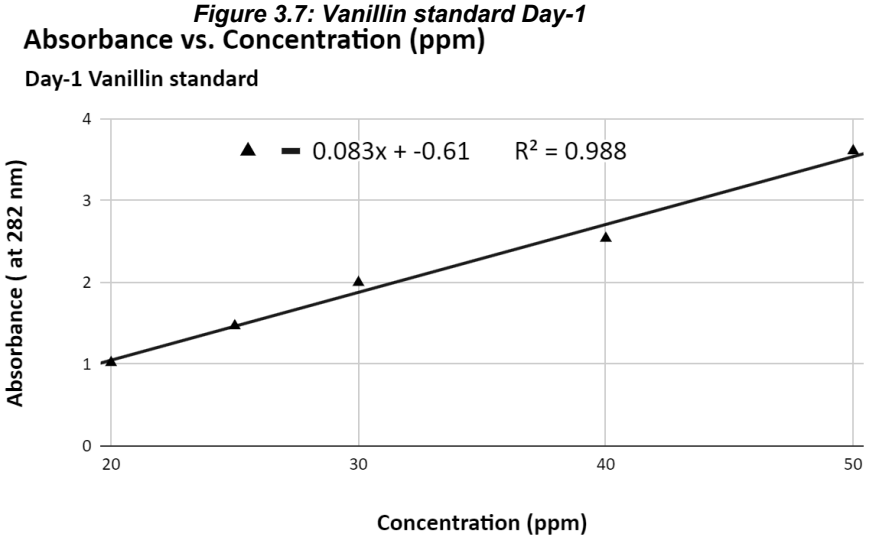
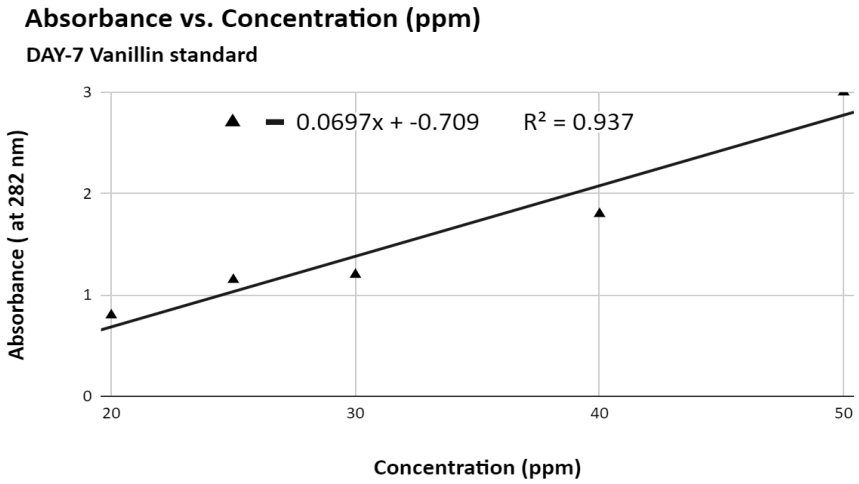


Figure 3.8: Vanillin standard Day-7

DAY-7

S No.	Concentration (ppm)	Absorbance (at 282 nm)
1	20	0.80
2	25	1.15
3	30	1.20
4	40	1.80
5	50	3.00

(Table 1.8 Vanillin standard)



D] PHENYLETHYLAMINE

DAY-1

S No.	Concentration (ppm)	Absorbance (at 257 nm)
1	5	0.019
2	10	0.037
3	15	0.38
4	20	0.45
5	25	0.57

(Table 1.8 PEA standard)

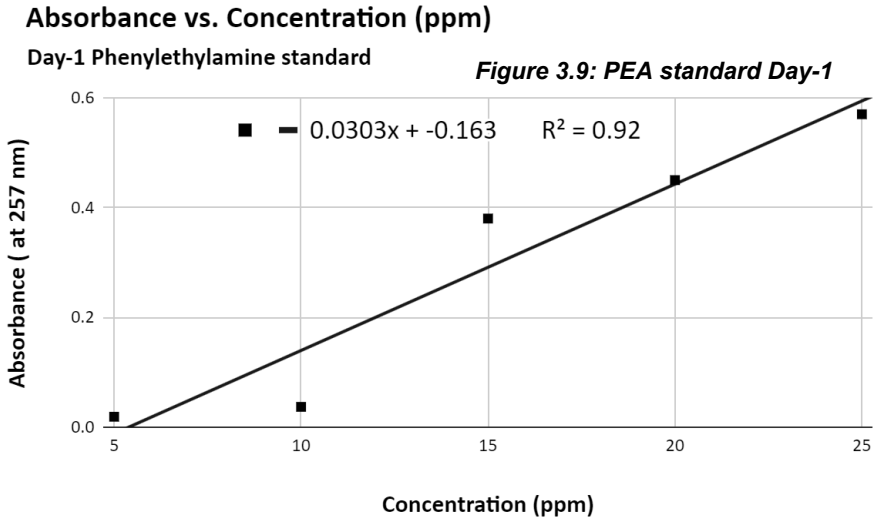


Figure 3.9: PEA standard Day-1

DAY-7

S No.	Concentration (ppm)	Absorbance (at 257nm)
1	5	0.0071
2	10	0.0237
3	15	0.0244
4	20	0.032
5	25	0.042

(Table 1.9 PEA standard)

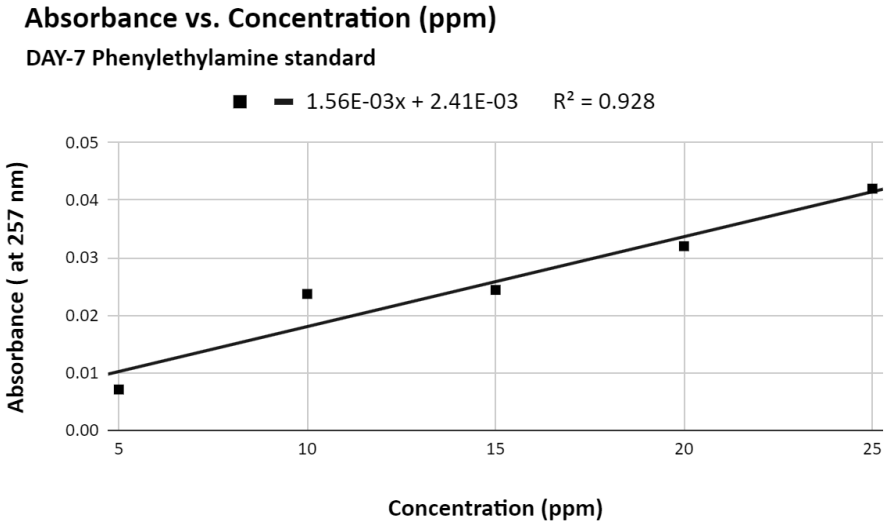


Figure 3.10: PEA standard Day-7

The next stage was using an HPLC-MS instrument (High-Performance Liquid chromatography combined with Mass spectrometry) in HPLC mode solely to run these standard solutions (20 ppm of each standard). The objective was to observe the strong peak and its duration of retention. The literature research revealed that a thorough method development is necessary for the greatest outcomes. Various mobile phase combinations were used throughout the course, for each standard's wavelength detection. The column used was a 5µm C-18 with dimensions of 4.6x150mm. The column was reversed-phase, with Octadecyl groups as the stationary phase and high-purity porous spherical silica as the base material. The column temperature was maintained at 40°C and 10µL of the sample was injected. The chromatograms below demonstrate method development done throughout the course:

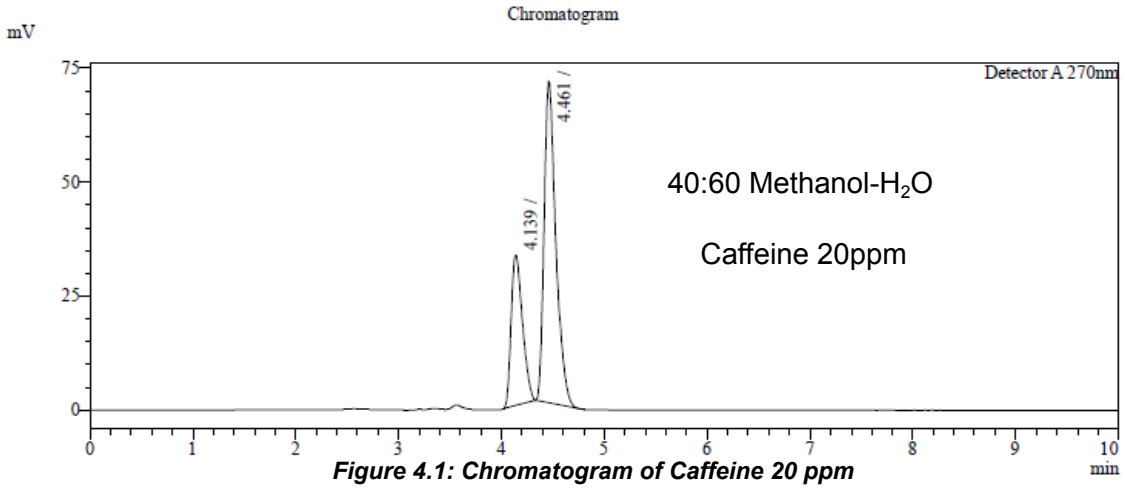


Figure 4.1: Chromatogram of Caffeine 20 ppm

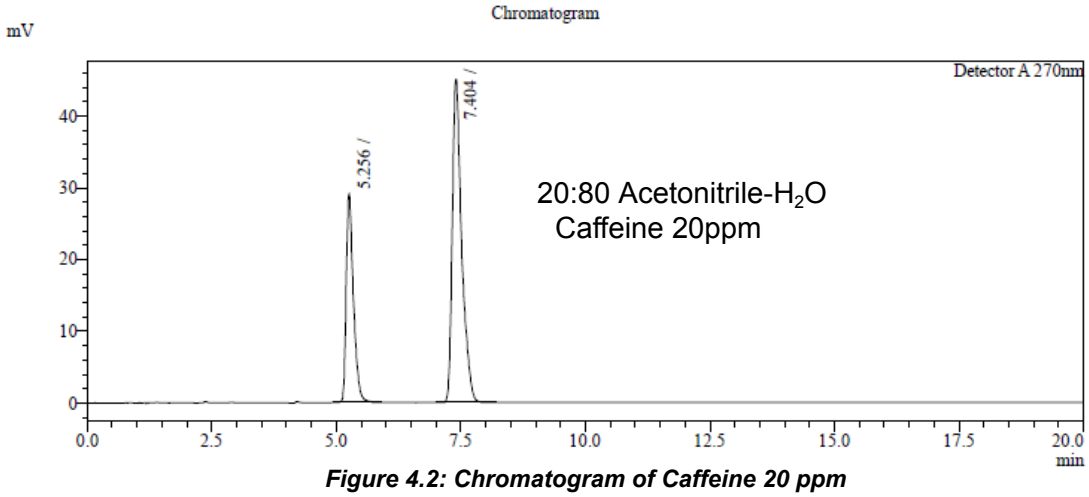


Figure 4.2: Chromatogram of Caffeine 20 ppm

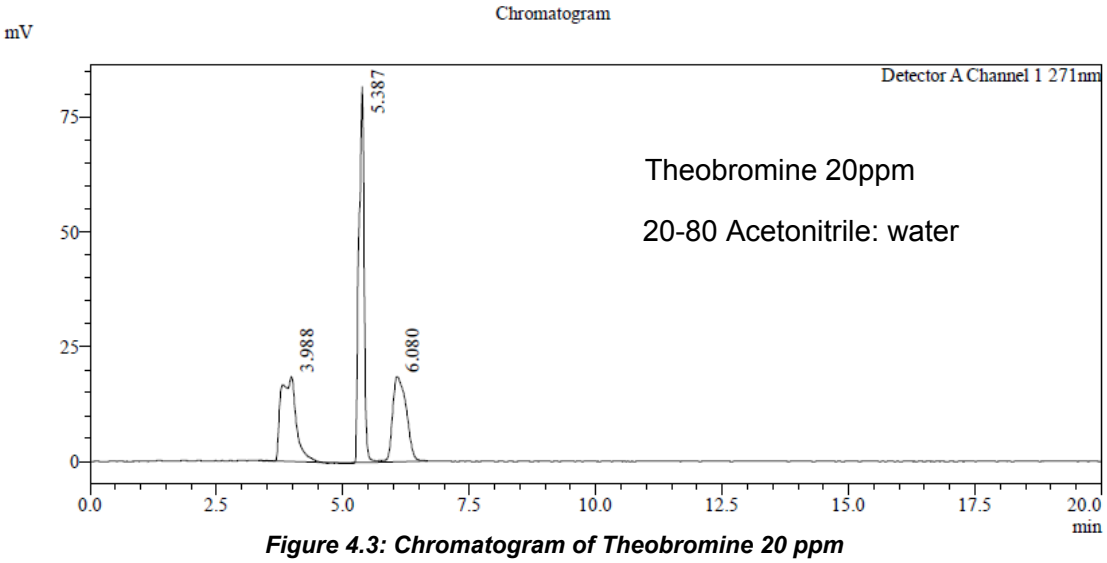


Figure 4.3: Chromatogram of Theobromine 20 ppm

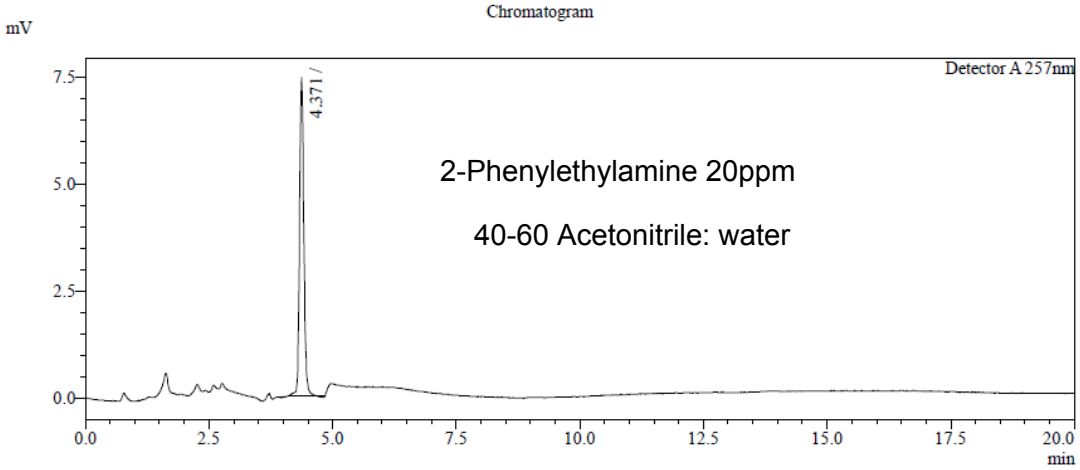


Figure 4.4: Chromatogram of Phenylethylamine 20 ppm

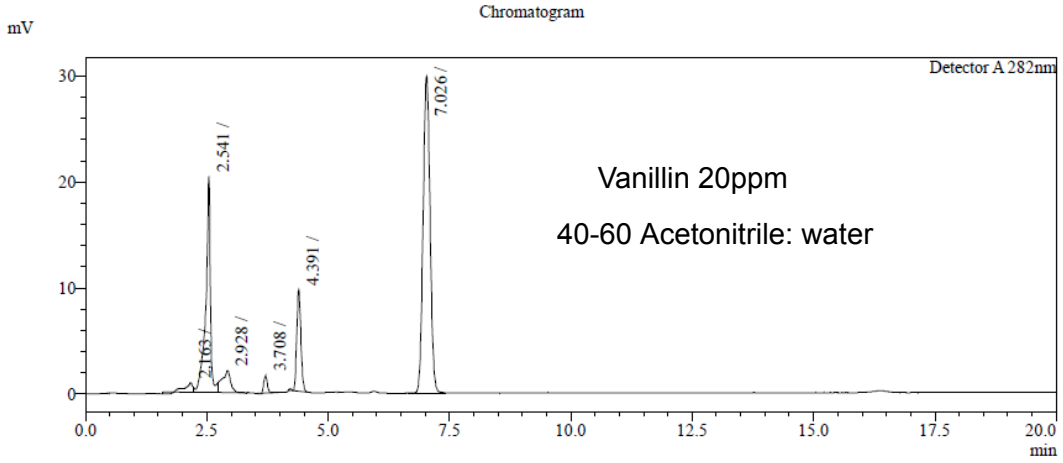


Figure 4.5: Chromatogram of Vanillin 20 ppm

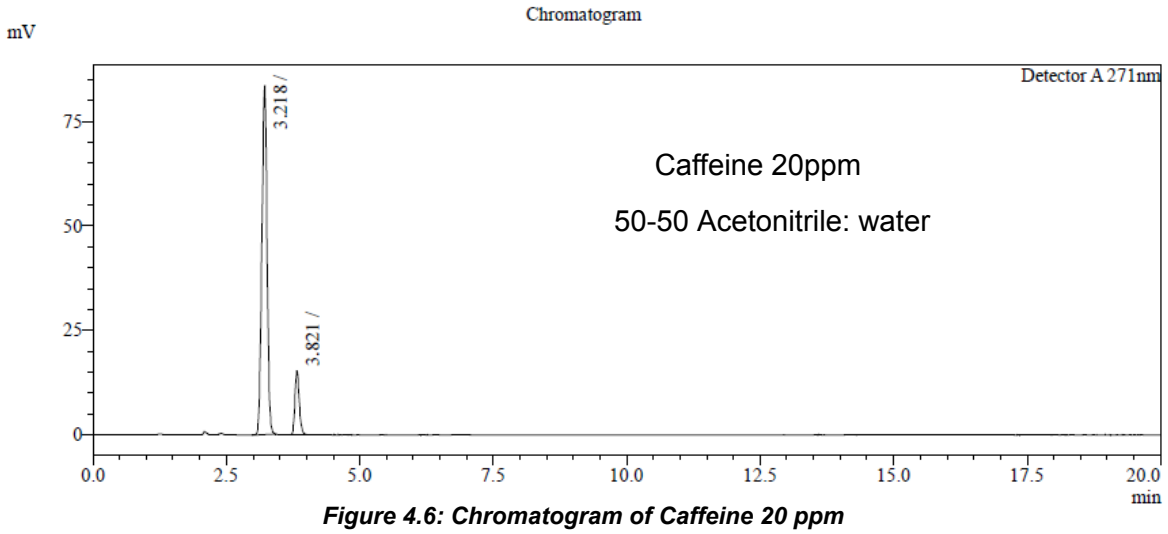


Figure 4.6: Chromatogram of Caffeine 20 ppm

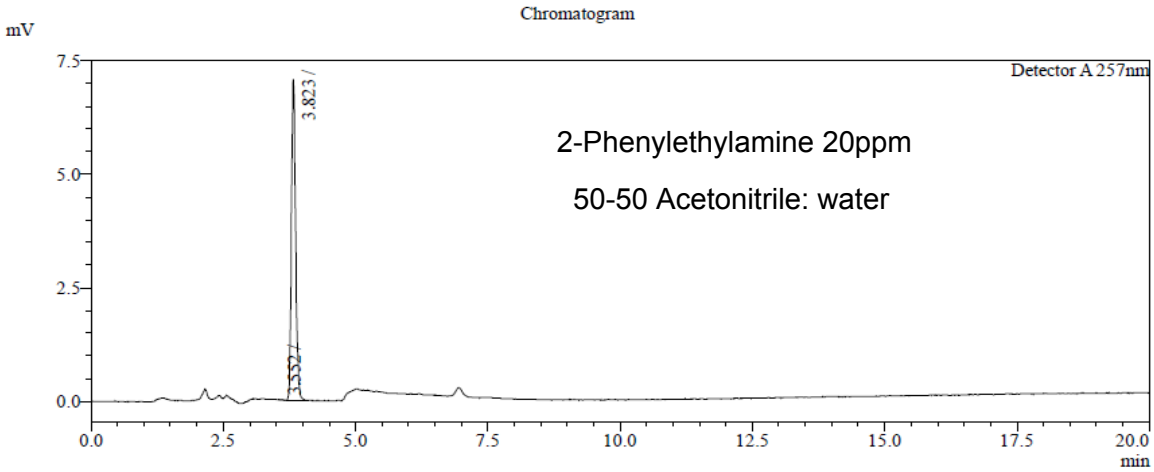


Figure 4.7: Chromatogram of Phenylethylamine 20 ppm

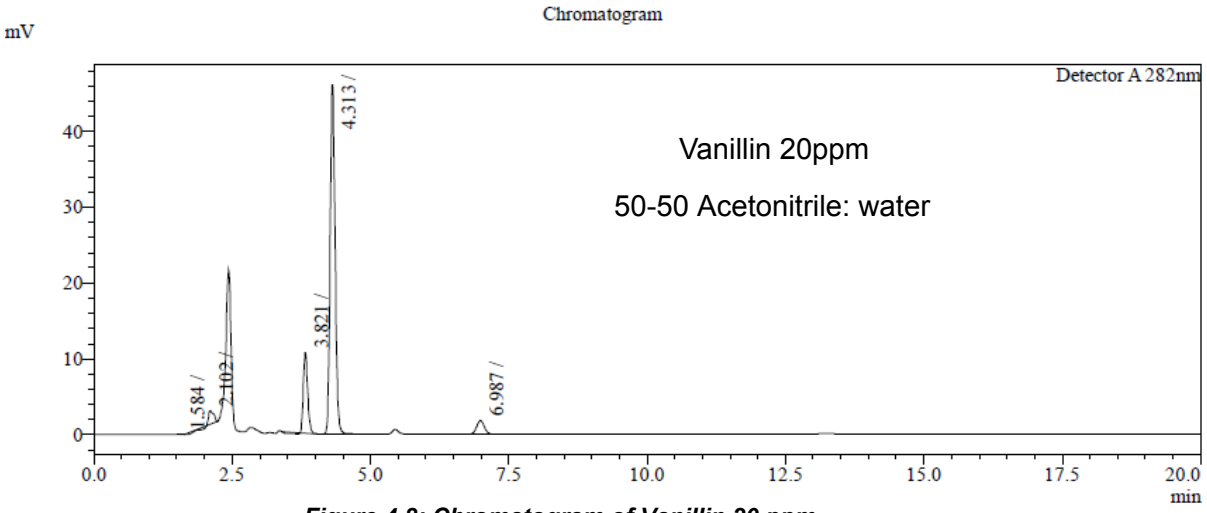


Figure 4.8: Chromatogram of Vanillin 20 ppm

The path of method development can be deduced from these chromatograms. Numerous combinations of mobile phases were tested, and it was discovered that 20:80 acetonitrile-water (both HPLC grade) produced the best possible results when numerous criteria, such as peak resolution and accuracy, were considered. It was crucial to note that theobromine and vanillin had numerous peaks, whereas caffeine had two peaks and 2-phenylethylamine had a single peak. As a result, it was decided to investigate the possibility of such outcomes. A higher concentration of caffeine was run on the instrument to see if there were still two peaks. However, just one distinct peak was found, implying that contamination occurred during the preparation phase/ equipment contamination/solvent contamination/vial contamination. Consequently, the apparatus was carefully cleansed with distilled water and then with HPLC-grade water before being placed in an oven at 60°C for 2 hours to remove any volatile/non-volatile contaminants. The analysis was performed using 20:80 acetonitrile-water at 271 nm with a flow rate of 1.5 mL/min. The column temperature was maintained at 40°C and 10µL of the sample was injected. Caffeine had a positive result with one strong peak, whereas vanillin and theobromine continued to display many peaks.

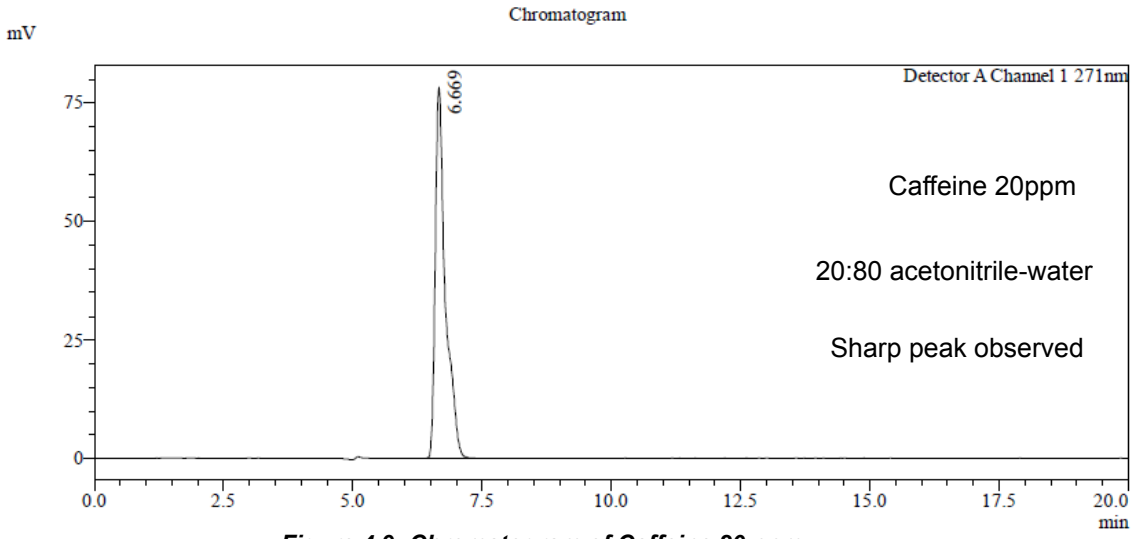


Figure 4.9: Chromatogram of Caffeine 20 ppm

Following the analysis, a mixture of all four standards (0.2mL each in a single vial) was run on the instrument to obtain clarity and differentiation between peaks in the presence of each other.

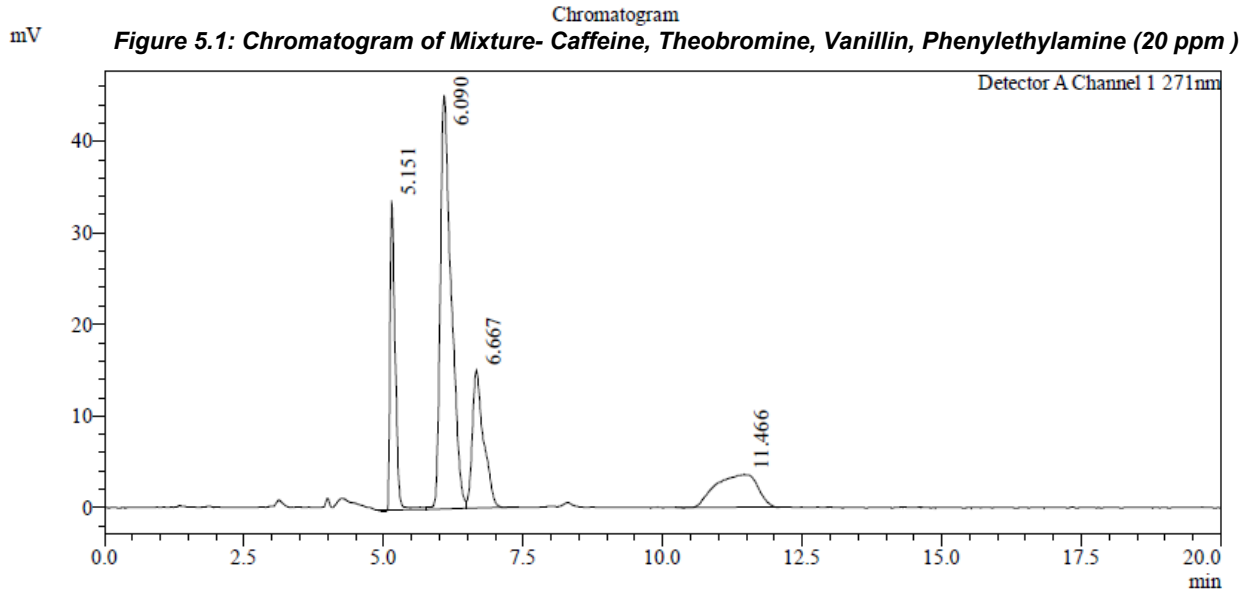


Figure 5.1: Chromatogram of Mixture- Caffeine, Theobromine, Vanillin, Phenylethylamine (20 ppm)

Retention duration of 6.667 min, 6.090 min, and 5.151 min were deduced to correspond to caffeine, 2-phenylethylamine, and theobromine, respectively.

Also based on this analysis, it was determined to test caffeine extraction from the sample first due to various factors such as its month-long stability, the strong peak seen, and its independence from using any buffers. Other standards could be discovered through data comparison, mass-to-charge ratio values, and spiking investigations.

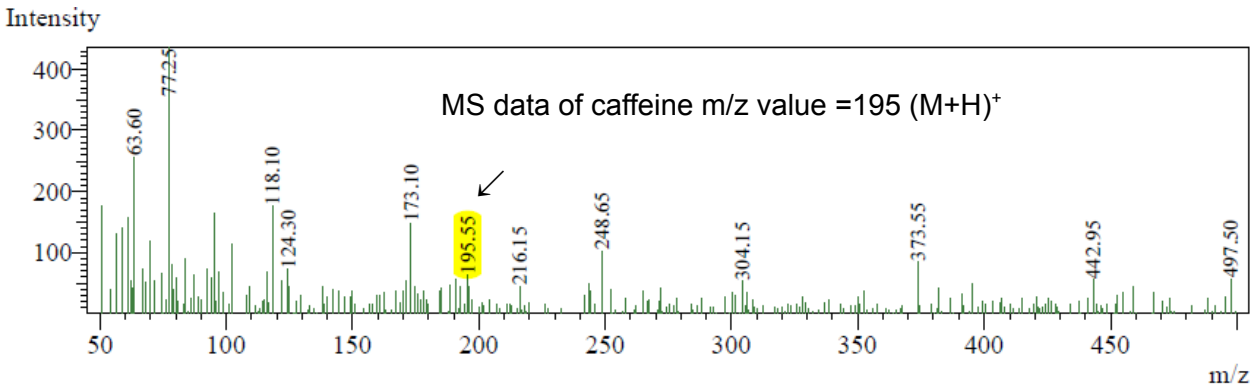


Figure 5.2: Mass Spectra of Caffeine

Following that, a series of caffeine standards of 5ppm, 10ppm, 20ppm, 40ppm, and 80ppm were prepared from a 100ppm stock solution. Filtered solutions were transferred to vials. Each concentration was replicated three times. The goal was to assure precision and accuracy, detect systematic mistakes, ensure reproducibility, and build a calibration curve which is then used to calculate the amount of analyte in a sample.

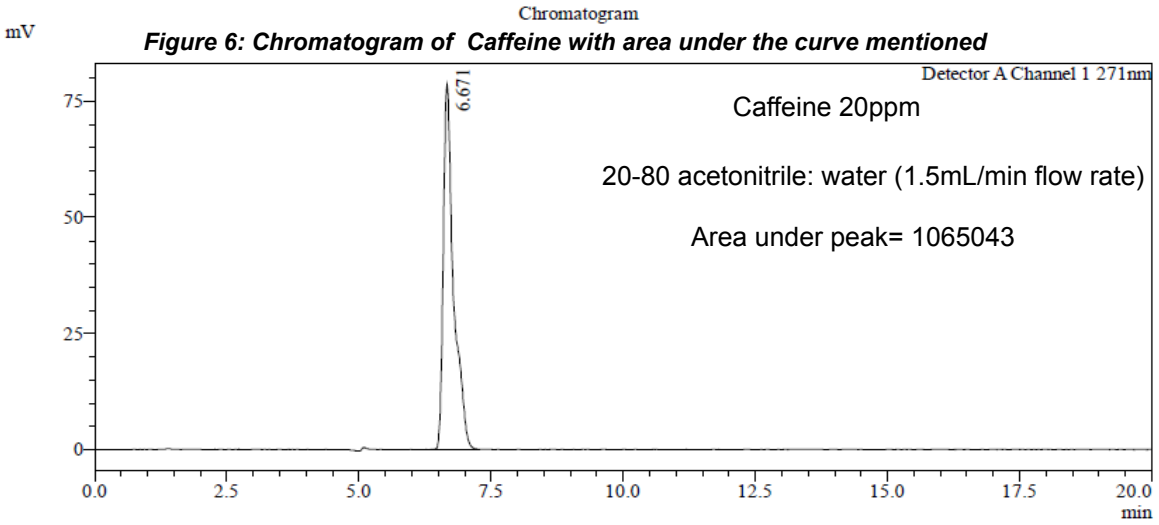


Figure 6: Chromatogram of Caffeine with area under the curve mentioned

Peak Table

Detector A Channel 1 271nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	6.671	1065043	78620	100.000		M	
Total		1065043	78620				

Similarly, for the calibration curve, all 15 chromatograms (five concentrations with three replicas) were interpreted, and the following table was created:

Table 2: Calibration curve of series of Caffeine standards

S.No.	Concentration (ppm)	Area under the peak 1	Area under the peak 2	Area under the peak 3	Area under the peak (Mean)
1	5	293199	292671	291983	292618
2	10	554604	553622	552054	553427
3	20	1072184	1065043	1067777	1068335
4	40	1421223	1418253	1414166	1417881
5	80	3876869	3908343	3865330	3883514

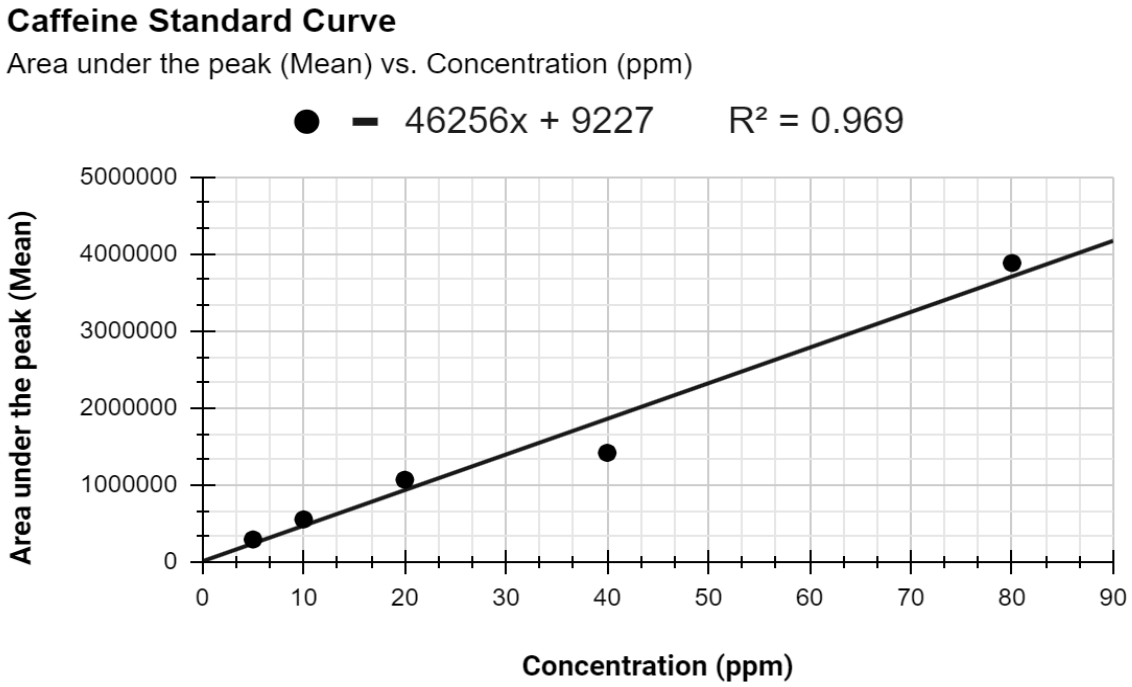


Figure 7: Calibration curve for Caffeine

The residual standard error for the regression line is 290138.1844

Hence the standard deviation obtained is 648768.7032

The next goal was to undertake sample preparations and identify target compounds after knowing the stability of standards, method reliability, reproducibility of standard solution response, good accuracy from R^2 value (0.97), and good precision.

The samples prepared were from milk chocolate, cocoa powder, green tea, coffee beans, and cocoa beans. Following was the procedure followed:

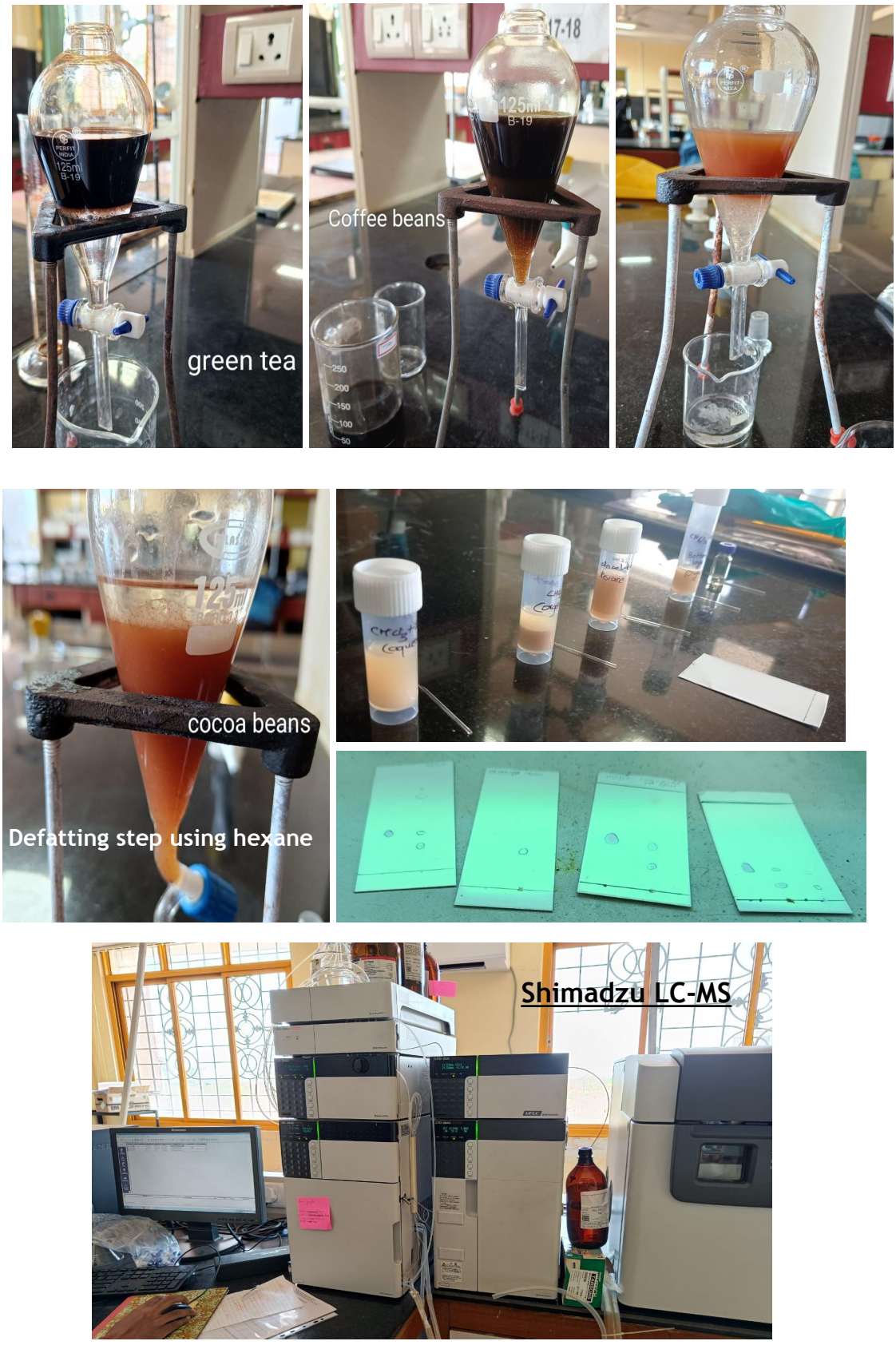
- An analytical balance was used to accurately weigh a small amount of chocolate sample, 4.2297 g
- The chocolate was grated and passed through 8 mesh screens to remove larger particles and bring uniformity.

- A suitable volume (~150mL) of HPLC-grade water (extraction solvent) was added to the chocolate sample in a 150 mL beaker and it was kept in a water bath at 100°C for approximately 30 minutes.
- After that, the beaker was withdrawn from the water bath and allowed to settle to form two different layers: the chocolate solid layer and the aqueous layer.
- For liquid-liquid extraction, the aqueous layer was decanted into a clean, dry separatory funnel. The extract was first defatted by adding 10mL of hexane solution. The separatory funnel was gently shaken twice before releasing the pressure by opening the nozzle. It was then rested on a tripod platform with the stopper removed. Two different layers were visible: the hexane (organic) layer above and the aqueous layer below.
- The organic layer was collected in a different beaker from the aqueous layer, which was collected in a separate clean, dry beaker. The aqueous layer was then put back into the separatory funnel, and 10mL of hexane was added again. The aqueous layer was similarly collected in a clean, dry beaker.
- This defatted aqueous layer was transferred to a new clean, dry separatory funnel.10mL of Dichloromethane (DCM) was added to this, and the separatory funnel was gently shaken to release pressure by opening the nozzle. The stopper was then removed, allowing it to rest. Two layers were seen, with an aqueous layer on top and an organic layer on the bottom (the layer of interest). The organic layer was carefully removed and transferred to a clean, dry beaker. In the meantime, another 10mL of DCM was added to the aqueous layer remaining in the separatory funnel. Similarly, the extraction was repeated three times, with the organic layer collected in the same beaker each time.

- To the organic layer in a beaker, 4 to 5 g of anhydrous sodium sulphate were added and thoroughly mixed to ensure free movement. This was done to get rid of any aqueous (water) residue. The solution was then filtered and collected in a round bottom flask before being evaporated with Rota-Vapour. Then there was a mixture obtained.
- The concentrated mixture was then dissolved in a suitable solvent (HPLC grade water mostly) and the solution was either transferred to a 100 mL standard flask to dilute up to the mark or was directly transferred to HPLC vials after filtering the liquid extract with a syringe filter to eliminate any leftover solid particles or contaminants.
- TLC (thin layer chromatography) tests were performed with care, spotting the sample and standard caffeine solution in a solvent mixture of 5% methanol in chloroform. The solvent system was chosen after extensive trial and error to produce sufficient resolution between the spots.
- The samples were collected in HPLC vials and then injected into the HPLC instrument to generate the chromatograms. Using a guard column in addition.

The samples made from cocoa powder and cocoa beans (finely powdered) (10.0171 g in 200 mL HPLC-grade water) were prepared in the same way. The sample preparation from green tea (8.3424 g in 200 mL HPLC-grade water) and coffee beans (finely powdered) (10.0001 g in 250 mL HPLC-grade water), however, did not include the defatting step; instead, a small amount of magnesium carbonate was added to the hot aqueous layer to remove tannins, and this hot aqueous layer was then filtered to transfer in a separatory funnel for extraction using DCM solvent. Figure 8 of experiments are listed below.

Figure 8: *Extractions of green tea, grounded coffee beans , grounded cocoa beans and LC-MS*



CHROMATOGRAMS AND DATA

Chromatograms of samples under the same method development:

Mobile phase- 20:80 acetonitrile-water isocratic

Flow rate- 0.4mL/min (Mass spectrometer connected)

Column temperature- 40℃

Time run - 20 minutes

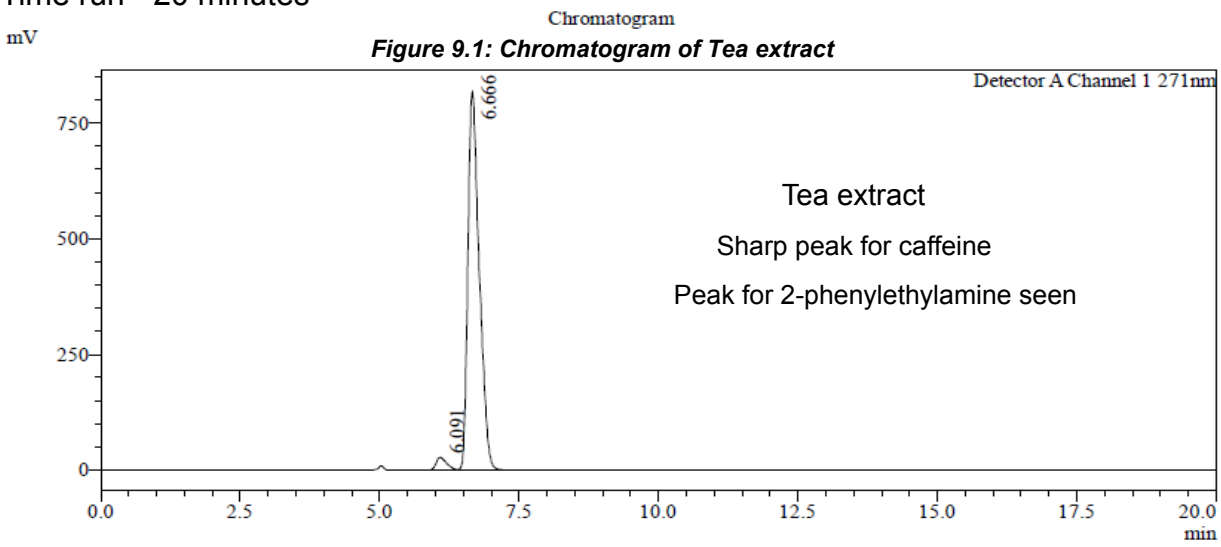


Figure 9.1: Chromatogram of Tea extract

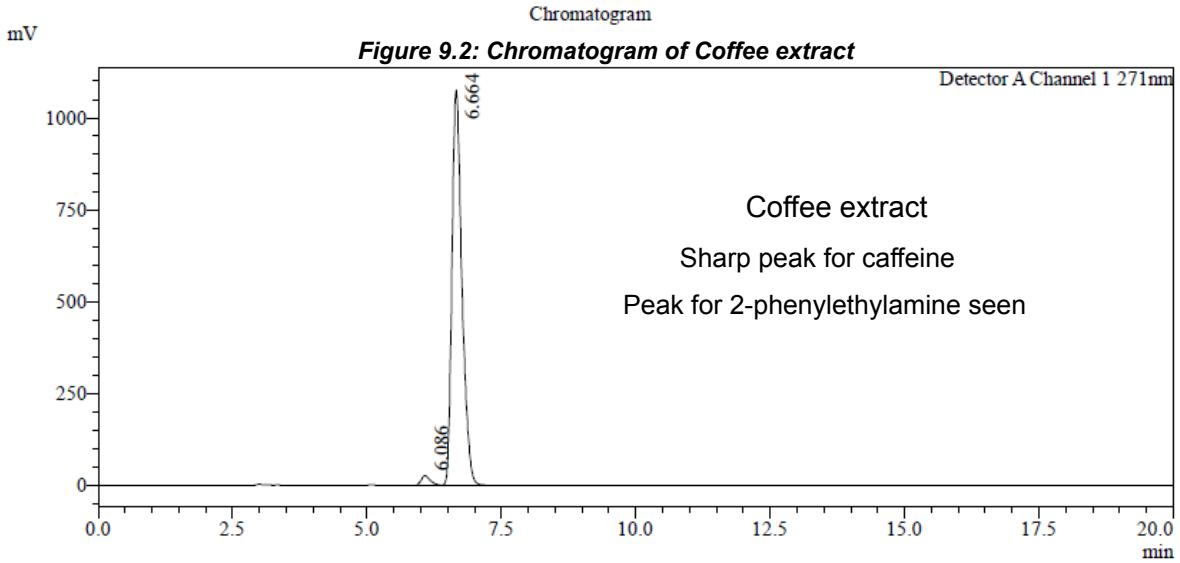


Figure 9.2: Chromatogram of Coffee extract

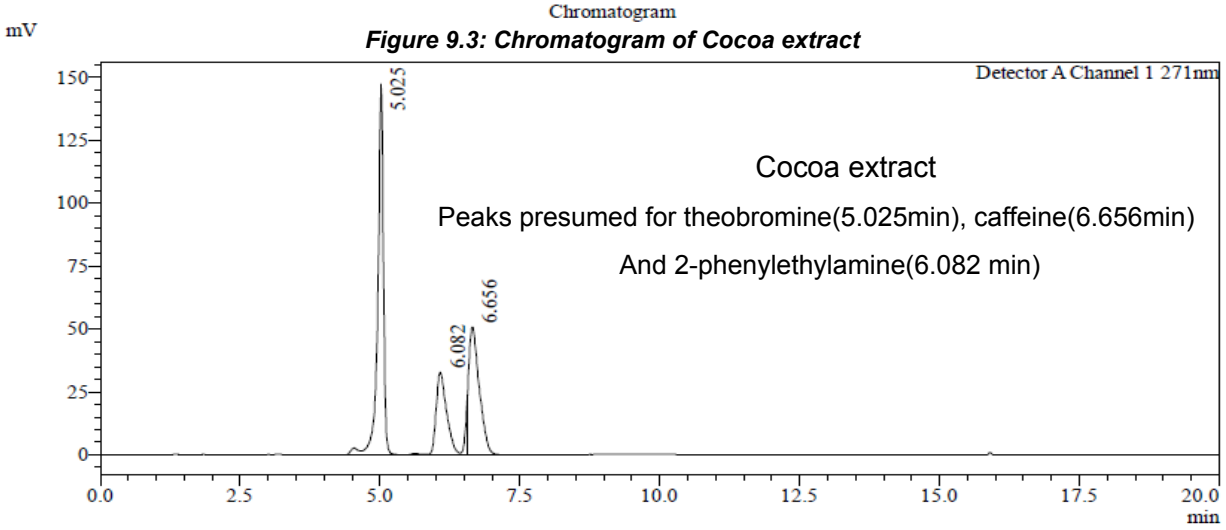


Figure 9.3: Chromatogram of Cocoa extract

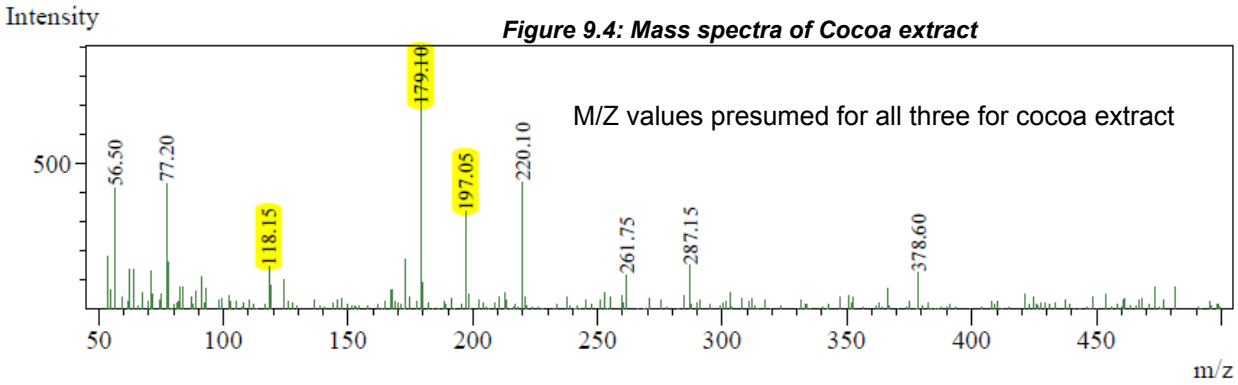


Figure 9.4: Mass spectra of Cocoa extract

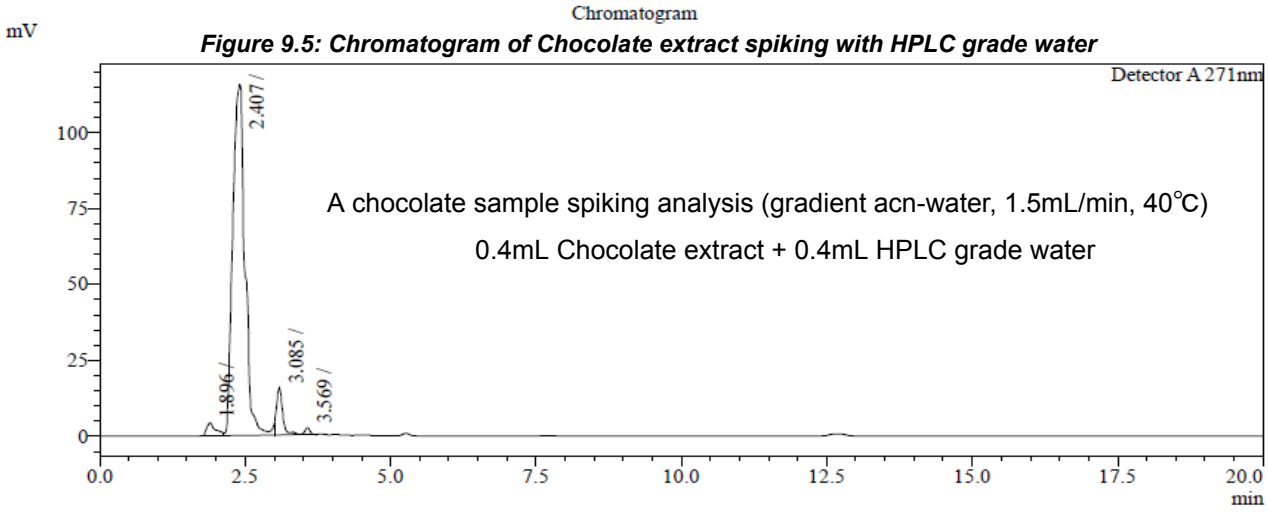


Figure 9.5: Chromatogram of Chocolate extract spiking with HPLC grade water

Peak Table CH-1

Detector A 271nm

Peak#	Ret. Time	Name	Area	Area%
1	1.896		44585	2.401
2	2.407		1686090	90.784
3	3.085		113869	6.131
4	3.569		12719	0.685
Total			1857263	100.000

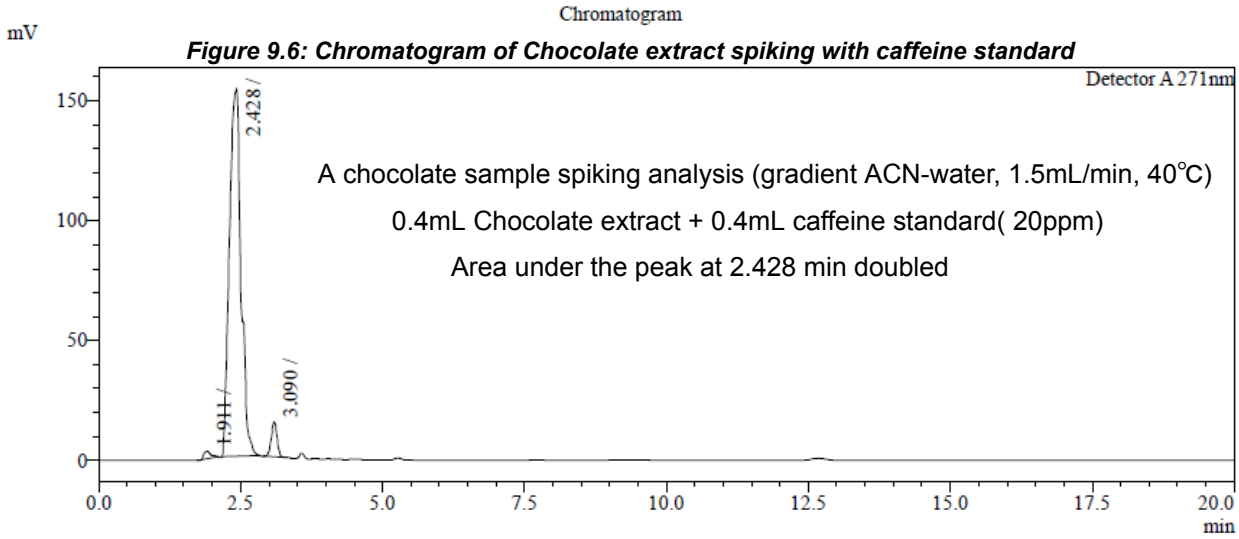


Figure 9.6: Chromatogram of Chocolate extract spiking with caffeine standard

Peak Table CS-2

Detector A 271nm

Peak#	Ret. Time	Name	Area	Area%
1	1.911		27451	1.192
2	2.428		2172797	94.334
3	3.090		103062	4.475
Total			2303311	100.000

Similarly, cocoa spiking experiments revealed the presence of theobromine, caffeine, and 2-Phenylethylamine. However, coffee spiking investigations confirmed the presence of caffeine and 2-Phenylethylamine.

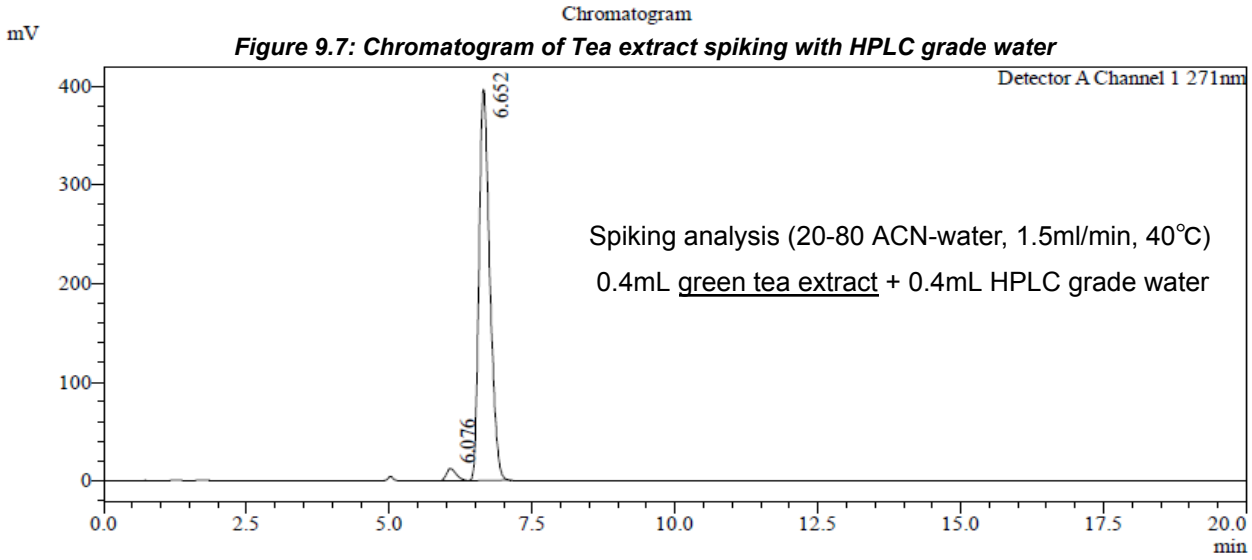


Figure 9.7: Chromatogram of Tea extract spiking with HPLC grade water

Peak Table

Detector A Channel 1 271nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	6.076	144548	12268	2.699		M	
2	6.652	5211457	396738	97.301		V M	
Total		5356005	409007				

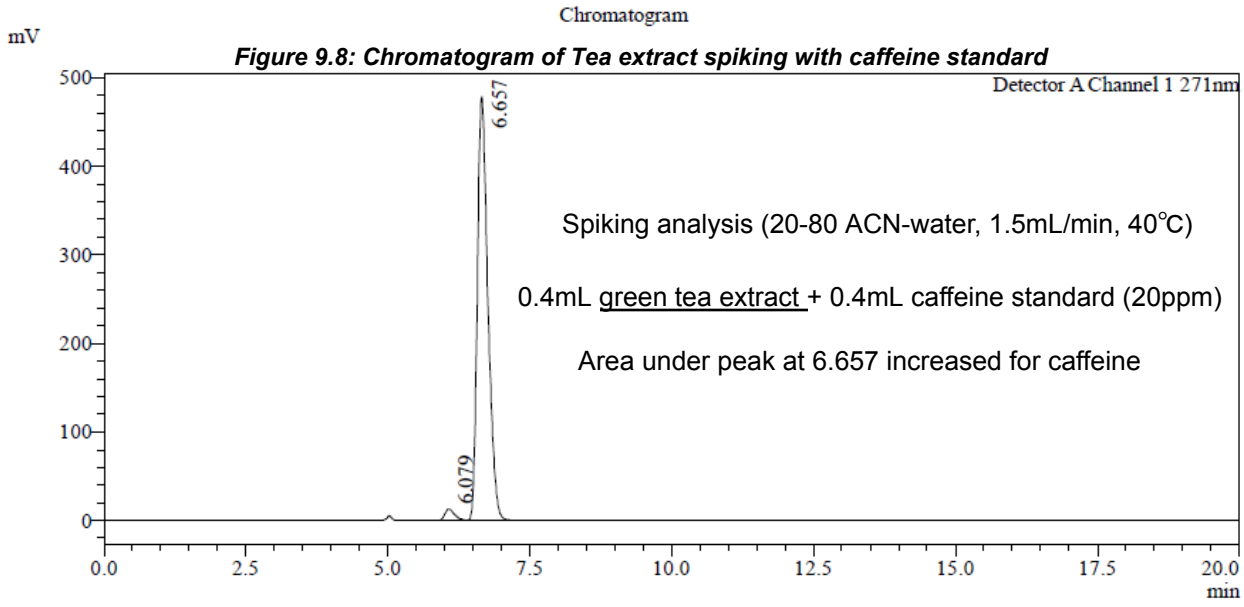


Figure 9.8: Chromatogram of Tea extract spiking with caffeine standard

Peak Table

Detector A Channel 1 271nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	6.079	153969	12988	2.432		M	
2	6.657	6177703	478020	97.568		VM	
Total		6331672	491007				

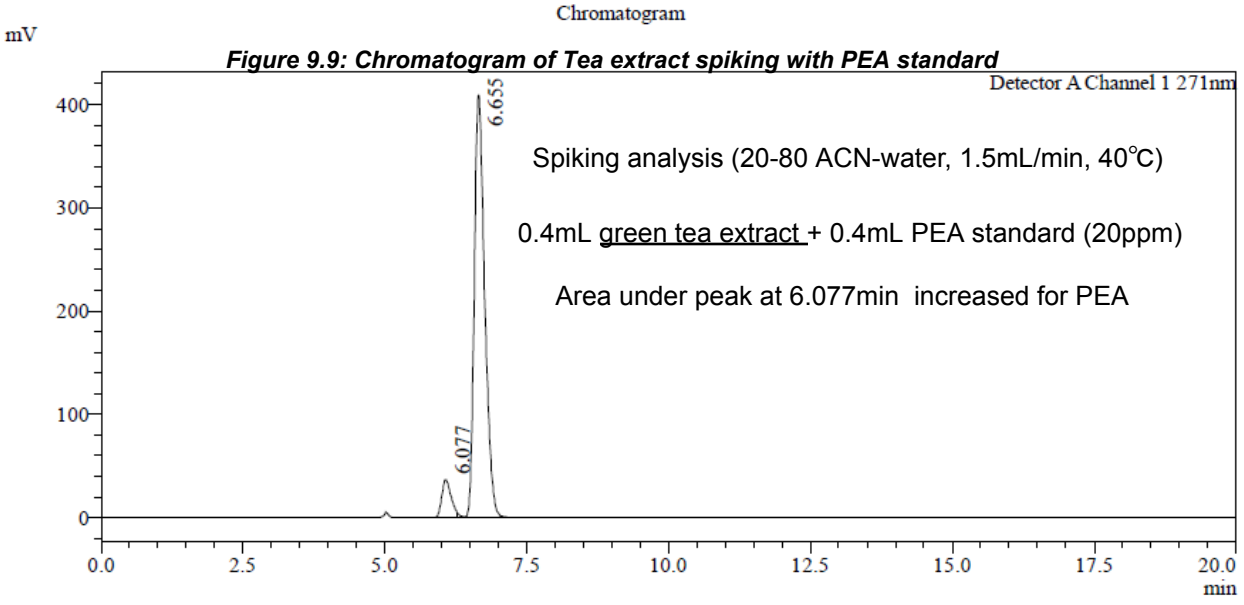


Figure 9.9: Chromatogram of Tea extract spiking with PEA standard

Peak Table

Detector A Channel 1 271nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	6.077	422675	36809	7.453		M	
2	6.655	5248373	409008	92.547		VM	
Total		5671049	445817				

Estimating unknown concentration of sample using a calibration curve prepared:

Table 3: Average area under the peak of sample extracts

SAMPLE	Area under the peak 1	Area under the peak 2	Area under the peak 3	Area under the peak (Mean)
GREEN TEA EXTRACT	11620402	11616649	11600465	11612505
COCOA EXTRACT	686240	652316	706827	681794
COFFEE EXTRACT	13854829	13927784	13797764	13860126

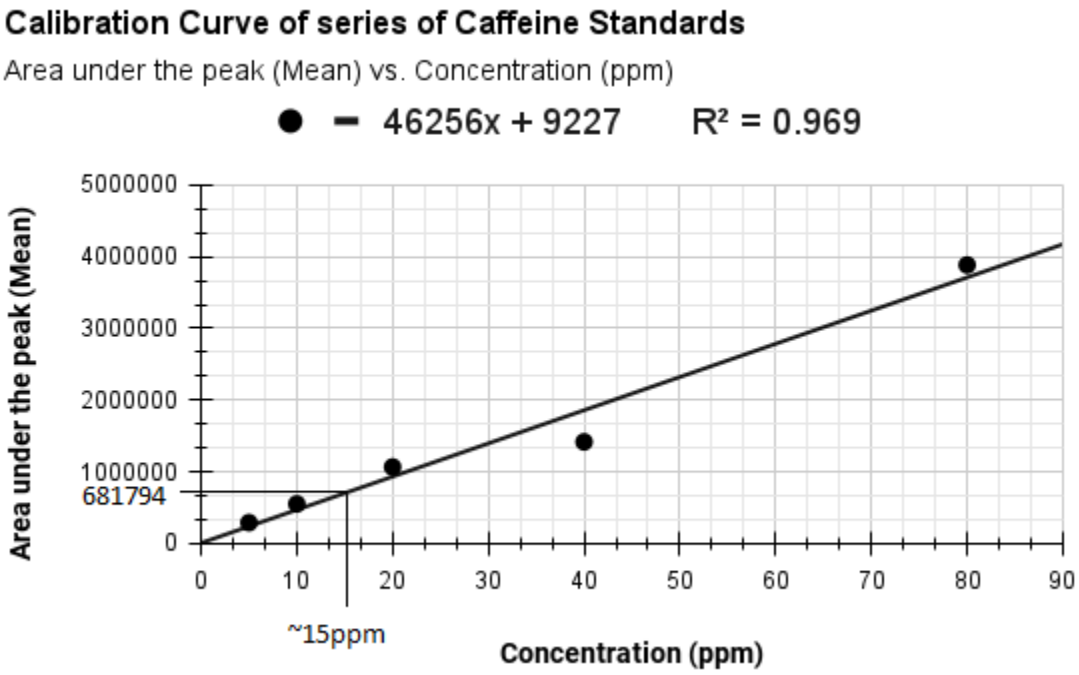


Figure 10: *Estimation of the concentration of cocoa extract taken(~75mL extracted from 10g dissolved in 250mL solution of HPLC-grade water)*

RESULTS AND DISCUSSION

This section presents a comprehensive analysis of the data collected in the study, focusing on the key findings and their significance in relation to the research objectives.

I. Stability test of Standards- The purpose of the stability test was to see if the concentration of the standard solution remained constant over time or if it degraded or changed in any way that could affect the accuracy and reliability of the analytical results. According to the UV-Vis spectrophotometer examination, Caffeine and Theobromine absorbance measurements did not change even after one month, indicating the good stability of these standards at ambient temperature. Vanillin and 2-phenylethylamine absorbance readings, on the other hand, fluctuated dramatically, indicating poor stability at ambient temperature. Stability is affected by a number of factors, including storage conditions and temperature. Each plotted graph depicts linearity and precision by displaying the value of R^2 (coefficient of determination). It demonstrates how well the data fit the regression model. R^2 values greater than 0.95 are generally considered to be accurate.

The R^2 value for caffeine and theobromine was adequate and steady for one month. It can be deduced that these standard solutions will be valid for usage for the duration of their indicated shelf life. However, the R^2 value for vanillin declined dramatically after a week, and the absorbance values for 2-phenylethylamine varied significantly within a week. As a result, it was deduced that these standards must be prepared from scratch for each analysis as degradation or alterations in the standard solution may result in erroneous and unreliable analytical data, which might have significant implications in analytical testing.

II. **Standards analysis on LC instrument:** Following several mobile phases, it was discovered that 20:80 acetonitrile-water in isocratic elution produced good results. It was found that theobromine and vanillin standards were not pure enough to do extractions from samples, but a chromatogram comparison could be performed to confirm their presence in samples. Multiple peaks in a chromatogram for theobromine and vanillin standards could be due to various factors such as structural isomers, stereoisomers, contaminants, and the chromatographic column used for separation, among others. Deep introspection resulted in a significant caffeine peak, and much knowledge was collected regarding probable contaminations at varying levels of preparation. Caffeine's mass spectrum was confirmed by the presence of m/z value =195 $(M+H)^+$

III. **The caffeine standard solution calibration curve:** was constructed by producing a graph of the Area under the peak (mean) vs standard solution concentration (ppm) to calculate the amount of analyte in a sample. A series of concentrations were created, and each was replicated three times to obtain the calibration curve. The solutions were filtered before being transferred to vials for analysis on the LC instrument. By determining the standard deviation of the response, which offers a measure of the precision and accuracy of the analysis, duplicates of standard solutions are employed to minimize the impact of random mistakes in the analytical procedure. It is possible to assess the instrument's performance and the method's stability by analysing replicates of standard solutions at regular intervals.

IV. **During the sample preparation:** of chocolate and cocoa powder, it was discovered that many emulsions formed, resulting in poor layer separation during solvent extraction. Many extraction experiments were conducted with various defatting

solvents and organic solvents (e.g., chloroform, a combination of chloroform and DCM) but adequate results were not obtained. Because of emulsions, a significant volume of extracting organic solvent was used without adequate separation. The finding was that the analytes of interest were partially soluble in both the aqueous and organic layers due to emulsions. A saturated sodium chloride solution was also used in the process to separate the emulsion, although the results were not favourable. It concluded that the complex matrix of chocolate and marketed cocoa powder required many improved and expensive solvents. As a result, spiking research was conducted instead. Caffeine was detected in the spiking research because the peak area of the spiked sample doubled. Whereas the sample preparation from tea, coffee beans, and cocoa beans was flawless. The layers separated clearly, and the chromatogram revealed prominent sharp peaks. This suggests that the chocolate product and cocoa powder had complex matrices such as preservatives, emulsifiers, impurities, and so on, which hampered extraction and contributed to multiple indistinguishable peaks.

V. **Spiking studies:** The samples were also tested in three replicates. Spiking studies are carried out to examine the precision and accuracy of analytical methods for detecting and quantifying target analytes in complicated matrices. Caffeine was found in all samples tested during the spiking investigation. Given that green tea is typically thought of as a healthy beverage, the significant level of caffeine detected in green tea was surprising. By comparing the findings to that of corresponding reference chromatograms, traces of vanillin and 2-phenylethylamine were found in a chocolate sample.

CONCLUSION

The liquid chromatography method created for the examination of caffeine, theobromine, vanillin, and phenylethylamine in typical beverages and food products is a potential instrument for finding and measuring the levels of these substances from an analytical chemistry perspective. The methodology is useful for the detection of these chemicals in complicated matrices due to the method's excellent sensitivity, specificity, and accuracy. The study also showed how crucial it is to optimize the chromatographic conditions for the optimal separation and detection of the target substances. This study highlights the importance of further study in this field by offering insightful information about the development of analytical techniques for the detection of trace chemicals in food and drink samples.

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