JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

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M. Sc. THESIS

ON:

DETERMINATION OF CAFFEINE CONTENT AND PHYTOCHEMICAL SCREENING OF RAW AND ROASTED COFFEE BEANS OF BENCH SHEKO ZONE, SOUTHWEST ETHIOPIA

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JIMMA, ETHIOPIA

DETERMINATION OF CAFFEINE CONTENT AND PHYTOCHEMICAL SCREENING OF RAW AND ROASTED COFFEE BEANS OF BENCH SHEKO ZONE, SOUTHWEST ETHIOPIA

A THESIS SUBMITTED TO JIMMA UNIVERSITY COLLEGE OF NATURAL SCIENCES DEPARTMENT OF CHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

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MSc THESIS APPROVAL SHEET

We, the undersigned, member of the Board of Examiners of the final open defense by **Feleke Mesfin Zeleke** have read and evaluated his/her thesis entitled **"Determination of Caffeine Content and Phytochemical Screening of Raw and Roasted Coffee Beans of Bench Sheko Zone, Southwest Ethiopia"** and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree Master of Science in **Chemistry**.

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Declaration

I hereby declare that, this thesis entitled **"Determination of Caffeine Content and Phytochemical Screening of Raw and Roasted Coffee Beans of Bench Sheko Zone, Southwest Ethiopia"** and the work presented in it are my original work and has not been presented for a degree in any other university and that all sources have been appropriately acknowledged.

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List of abbreviation

UV-Vis	Ultraviolet Visible spectroscopy
FTIR	Fourier Transform Infrared spectroscopy
CA	Coffee Arabica
FC	Folin-Ciocalteu's
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometer
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
TLC	Thin Layer Chromatography
TMP	Traditional Medicinal Plants
LLE	Liquid-Liquid Extraction

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Abstract

Caffeine is a naturally occurring alkaloid which is found in the leaves, seeds or fruits of over 63 plants species worldwide. The most common sources of caffeine are coffee, cocoa beans, cola nuts and tea leaves. Caffeine is a pharmacologically active ingredient and depending on the dose, can be a central nervous system stimulant, improve cardiac performance, increase brain circulation, and exhibit vasodilator and diuretic effect.Therefore, the objective of this study is to determine caffeine content and phytochemical profiles of coffee beans of Benchsheko zone in south west region. The coffee beans were collected from four different areas of Benchsheko zone (Debrework, Sheko, Shebench and Mizan). The coffee beans were roasted and ground using manual grinder. Extraction of raw and roasted coffee beans was performed using H_2O:$CHCl_3$ for GC-MS analysis and it was carried out sequentially using petroleum ether, chloroform, acetone and methanol for phytochemical screening tests and bioassay. Gas Chromatography - Mass Spectrometry (GC-MS) method was validated for determination of the levels caffeine in raw and roasted coffee sample of Bench Sheko zone. Agilent 5977B GC/MSD column was used with water: chloroform 50:50 % (v/v) eluent. Linearity of the method was check from 1-10 μg/L and the correlation coefficient was 0.9946. The spiked recoveries for caffeine were in the range of 100.64% to 117.6% for the raw and 83.77% to 115.83% for the roasted coffee. The caffeine contents in coffee samples were 7.45 μg/L and 5.8 μg/L Mizan roasted and raw coffee;9.82 μg/L and 5.62 μg/L Debrework roasted and raw coffee; 8.01 μg/L and 4.94 μg/L for Shebench roasted and raw coffee;8.22 μg/L and 5.42 μg/L for Sheko roasted and raw coffee, respectively. Phytochemical screening revealed presence of alkaloids, phenolics, flavonoids and terpenoids in coffee beans.As it can be concluded from the results, 21.9 - 42.8% of the caffeine content of coffee beans increases upon roasting.

Keywords: Caffeine, Coffee Beans, Coffee Roasting, GC-MS, phytochemical screening

1. INTRODUCTION

1.1.Background of the study

Today, coffee is one of the most consumed beverages in the world mainly due to its stimulating effects, characteristic taste, and richness of coffee aroma which makes it a unique beverage. There has been identified more than 80 species of coffee in the world, but only two main coffee species: Coffea (Arabica), which stands for about 60% of the world coffee market, and Coffee canephora (Robusta) about 40%. Minor cultivated species include C. (Liberica) and C. excelsa account for only 1-2% of global production. Arabica and Robusta have a very distinct chemical composition and Arabica coffee is milder, more aromatic and contains less caffeine than Robusta coffee [1]. However, Robusta coffee tree is stronger and more resistant than Arabica tree in various aspects [2]. The different varieties of coffee show differences in the size and shape of the coffee bean but, on average, beans are approximately 9.5 mm long and 7 mm wide. The weight of a parchment seed at 9%moisture content is about 0.15 g for Arabica and 0.16 g for Robusta. The bean sizes are also influenced by environmental conditions and husbandry (nutrition, moisture, care etc.).

Coffee is a complex mixture of thousands of chemical compounds responsible of its flavor and aroma, carbohydrates, lipids, nitrogenous compounds, vitamins, minerals, alkaloids, and phenolic compounds [3]. Coffee is rich in many bioactive substances (trigonelline, chlorogenic acid, diterpenes, caffeine), and its consumption has been associated with many beneficial effects [4]. Coffee chemistry is determined by numerous factors, including the varietal of bean, region of origin, growing conditions, roasting process, grind size distribution, water chemistry, and temperature of water used during extraction [5].

Caffeine (1, 3, 7-trimethylxanthine) is known as a Central Nervous System (CNS) stimulant. It is a naturally found in the leaves, seeds and/or fruits of at least 63 plant species worldwide. The most commonly known sources of caffeine are coffee, cocoa beans, kola nuts and tea leaves[6]. Caffeine is consumed most frequently in beverage such as coffee (71%), soft drinks (16%), and tea (12%)[7]. Coffee beans contain between 0.8 and 2.8% caffeine, depending on species and origin. It

contributes to 10-30% of the bitter taste of coffee brews [8]. The caffeine content in C. canephora (Robusta) is about two times that of C. Arabica (Arabica) [9]. Caffeine content of coffee bean is not significantly changed during coffee roasting [2], Green coffee beans contain 5–14% of the reported major components, chlorogenic acids (CGAs)[2] and these compounds are significantly reduced after roasting [9]. Literature data indicated an inverse relation between roasting time and antioxidant activity due to the degradation of chlorogenic acids and other phenolic compounds [10]. Increase roasting time found to decrease both the anti-oxidant and anti-inflammatory potential of different coffee extracts [12]. However, the effect of roasting in caffeine content is controversial. The GC-MS indicated that caffeine contents are higher in the roasted coffee than the raw coffee [13]. Other researchers found that both phenolic and caffeine contents decrease in the darker grades of coffee [13]. Increases in roasting temperatures correlate to a decrease in extractable chlorogenic acid concentrations andan increase caffeine concentrations [8]. Thus, further study in the area need to be investigated. Hereby, quantitative analysis of caffeine and qualitative testwere evaluated for Ethiopian coffee samples collected from Bench Sheko zone.

1.2. Statement of the Problem

The stimulant effect,its pleasant taste, aroma and health benefits of coffee makes it one of the most popular beverages in the world is due to the presence of caffeine (1, 3, 7-trimethylxanthine). However, high doses of these compounds may produce negative effects in some sensitive individuals, including anxiety, tachycardia and insomnia. In addition to this, some of these compounds are thermally unstable and degraded during roasting and change the coffee's test and aroma. Therefore, it is important to study the caffeine content ofcoffee beans collected from different places of south west region.

1.3. Research Questions

This study answers the following questions:

1. Is the quantity of caffeine in coffee differsin different geographical location?
2. Is the quantity of caffeine differing between roasted and raw coffee?
3. Is roasting and extraction solventaffect the presence of phytochemicals in coffee beans qualitatively?

1.4. Objective of the Study

1.4.1. General objective

The main objective of this study is to assess the caffeine content and phytochemical profile of roasted and raw coffee beansof Coffee samples from Bench Sheko zone.

1.4.2. Specific objectives

- To determine the caffeine content of Coffee collected fromBench Sheko zone
- To compare and contrast the caffeine content of roasted and raw coffee beans
- To identify the phytochemical test inroasted and raw coffee beans

1.4.3. The Significance of the Study

The outcome of this study would have significant contribution to filling the current research gap on the caffeine content of roasted and raw coffee and phytochemical screening test of coffee beans. In addition, the results obtained from this study can be used by coffee traders, coffee processors and researchers as useful inputs when purchasing, using and studying caffeine content conditions. This data will be used as base line information to advance research knowledge in similar areas.

It will create an opportunity to develop the industry-university research institutes linkage to conduct applicable researches which will benefit both the researcher and the industry.

2. REVIEW OF LITERATURE

2.1.History and Economic importance of coffee

Coffee is most popular drink around the world. Different people of the world consume coffee in different forms. Historically, consumption of coffee had been related to unhealthy behaviors, such as smoking, large amount alcohol drinking. But recently it becomes popular due to its beneficial activities [15]. All cultivated species of coffee have their origin in Africa. Coffee is first found in Ethiopia, where shepherds discovered the coffee beans in the 6 centuries [15]. The Arabs introduced coffee from Ethiopia to Yemen during the 13th century, where the habit of drinking coffee was developed in the 15th century. This habit gradually spread to the rest of the world, leading to the increased interest of some countries to produce coffee as a commodity on a large scale.

Coffee has enormous commercial and social importance and is the most important traded commodity in the world after oil [16]. Global output is expected to reach 7.0 million tons by 2010. World consumption of coffee is projected to increase by 0.4% annually from 6.7 million tons in 1998–2000 to 6.9 million tons in 2010[16]. It is also very important commodity crop for many developing countries, once contributing over 10–11 billion US $ annually and providing a source of income for thousands of small-scale farmers, as well as being a significant source of employments [17]. The production of coffee beans is the base of the economy of several tropical countries, such as Mexico, Guatemala, El Salvador, Nicaragua, Costa Rica, Panama, Venezuela, Colombia, Ecuador, Peru, Bolivia, Paraguay, Brazil, Ethiopia, Kenya, Tanzania, Zambia, and Mozambique [17]. More than 50 countries, in which 25 of them in Africa, depend on coffee for their foreign exchange Ethiopia is the third largest coffee producer in Africa after Uganda and Ivory Coast [18]. It covers 2.5% of the world's coffee trade [19] Coffee covers about 60% of Ethiopia's export [19]. It also accounts for 5% of gross national product, 42% of taxes from foreign trade. In the country 24.5 Kg coffee is consumed annually per household and 4.5 Kg per capita [19].

Coffee grows in most part of Ethiopia. Oromia and SNNP regions comprise largest coffee cultivated areas such as Wolega, Elu Ababoura, Jimma, BenchMaji, Sidamo, Gedeo, Guji, East

and West Hararge, South and North Omo. About 204500to 683600 hectares of land is under coffee cultivation [20]. This is a small part of areas, which is suitable for coffee production. Around 25% of the country's population directly or indirectly depends on coffee [18]. About 95% of the coffee output is grown by small farmers, most of whom work less than half a hectare of land. An average yield is between 540 to 490 Kg per hectare [20].

2.2.Coffee Constituents

Coffee contains a multitude of substances, many of which are potentially biologically active. The chemical composition of coffee varies for different reasons such as the species and variety of coffee beans and to a lesser extent other factors such as agricultural activities, degree of maturation and storage conditions [19]. In the order of their abundance, coffee contains phenol polymers 8%, polysaccharides 6%, chlorogenic acids 4%, and minerals 3%, water 2%, and caffeine 1%, organic acids 0.5%, sugar 0.3%, lipids 0.2% and aroma 0.1% [18]. Organic acids such as oxalic, succinic, fumaric, malic, tartaric, citric and quinic acids are considered to play an important role in coffee flavor [21].

Coffee has the highest and the most variable caffeine content among dietary products, which contain this alkaloid. The value differs from 30-175 mg of caffeine per cup (150mL) coffee. The standard value has been suggested to be 85 mg of caffeine per cup of ground roasted coffee [22]. Coffee is also enriched with many other ingredients that may contribute to its biological activities such as niacin, potassium, magnesium and antioxidants such as tocopherols, phenols and chlorogenic acids. The two Di-terpinoids, cafestol and kahweol, are also found in significant level. They are a natural constituent of green coffee beans, are realized from roast, and ground coffee by hot water [21].

2.3.Caffeine

Caffeine (1, 3, 7-trimetylxanthine) is one of the main alkaloid found in various kinds of foods and drinks that we consume in daily life [23].It is naturally found in leaves, seeds or fruits of 63 plant species [23]. The most common sources of caffeine are coffee, cocoa beans, cola nuts and tea leaves. The chemical formula for caffeine is $C_8H_{10}N_4O_2$. Pure caffeine occurs as odorless, white powder. It has molecular weight of 194.19 g, melting point of 236 ^{o}c, and sublimation point of

178 °C and pH values in the range of 6 to 9 [22].

Figure 1. Caffeine in coffee

Based on the data reviewed, it can be concluded that low to moderate caffeine intake (300 mg/day or less) is generally associated with improvements in alertness, learning capacity, exercise performance and perhaps mood. In addition, caffeine metabolites, especial 1-methylxantine and 1-methylurate have exhibited antioxidant activity. Antioxidants have been widely linked to a number of potential health benefits including protection against heart disease and cancer. However, high doses may produce negative effects in some sensitive individuals, including anxiety, tachycardia and insomnia [24]. A study of Dr. Pollack [25] recommended upper limits for caffeine: healthy adults should consume below 300-500 mg daily, pregnant women must stay below 150-200 mg daily and children should stay below 50 mg daily.

2.3.1. Absorption and Metabolism

Caffeine enters into human body through different sources such as food stuffs, beverage and medications. Caffeine absorption from the gastrointestinal tract is rapid and virtually completed about 45 minutes after ingestion. The absorbed caffeine is readily disturbed throughout the entire body. It passes across the blood brain barriers, through the placenta into amniotic fluid and the fetus, and into the breast milk [26].

Caffeine is metabolized by demethylation and oxidation. It is metabolized in the liver through a series of N- demethylation and purine ring oxidation reaction to yield a mixture of mono and dimethylxanthines and methyl uric acids. The main dimethyl xanthine metabolites are theophylline (4 %), theobromine (12 %) and Para xanthine (1, 7-dimethylxanthine) (80 %). Para-xanthine is not found in foods but it is the main metabolite of caffeine; 1, 3, 7, trimethyl uric acid and 6-amino-5(N-formylmethylamino)-1,3dimethyluracil together account for 15% of caffeine elimination. The metabolism is slowed during pregnancy and in women taking oral contraceptives. On the other hand, the metabolism rate of caffeine is greater in smokers than non-smokers [26].

The half – life of caffeine, the time required for the body to eliminate one-half of the total amount of caffeine consumed at a given time varies widely among individuals according to such factors as response, age, sex, liver function, pregnancy, some concurrent medications, smoking and the level of enzymes in the liver needed for caffeine metabolism. Caffeine has a half - life which is faster than theobromine. It is completely metabolized by human body; only 1-5 % of ingested caffeine is recovered unchanged in the urine. Infant up to the age of 8-9 months have a greatly reduced ability to metabolize caffeine, excreting about 85 % of the administered caffeine in the urine unchanged [27].

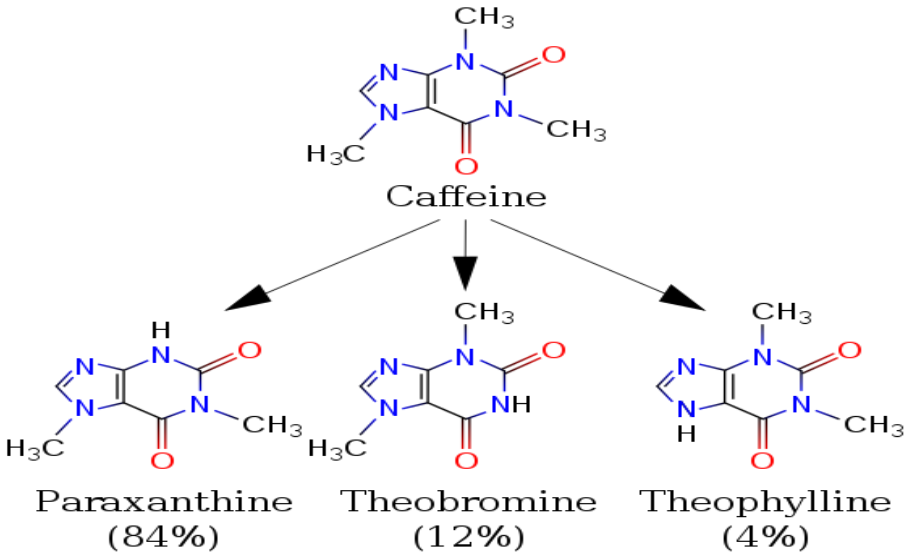

Figure2. Metabolism of caffeine in the liver

The half- life in the newborn child range from 5-100 hrs; But it gradually approaches that of an adult. By 6 months of age caffeine's half- life is about 20-30 % shorter in females than in males. The half-life in females using oral contraceptive steroids is approximately twice that observed for ovulatory females. Pregnancy slows down the metabolism of caffeine. The metabolic half –life increases steadily from 4 hrs during the first trimester to 18 hrs during the third trimester. Obesity also slows down caffeine metabolism. Smoking is another factor which affects the metabolism of caffeine. It accelerates the rate at which caffeine is eliminated [27].

2.3.2. Beneficial Effects of Caffeine

Caffeine is a plant based alkaloid which stimulates the central nervous system. The stimulatory effects of caffeine usually result in an increased ability for mental activity for sweets by simulating the production of those adrenal hormones that cause blood sugars to be increased. Stimulatory effects of caffeine results in increased capacity for mental activity and muscular work, the weakness, depression and discomfort from excess of alcohols can be nullified with black coffee or hypodermic injections of caffeine. Insensibility from hashish is believed to be ended by the use of caffeine medication. Even the dullness and sense of depression from a little too much tobacco is helped by coffee [28].

The other benefits of caffeine include reduced risks of Parkinson's disease, colon cancer, diabetes; decrease in exercise induced myocardial flow reserve and increase and in both sexual motivation and locomotors activity on female mating behavior. Caffeine expands blood vessels and consequently the brain receives more oxygen. It helps in preventing a positive energy balance and obesity. Caffeine is also an accepted drug for intra muscular application to treat arterial hypotension [29].

2.3.3. Physiological Effects of Caffeine

Caffeine is a powerful central nervous system stimulant; a mild diuretic and has been used as a mild anti-depressant. It has many physiological effects such as, gastric and secretion, dieresis and stimulation of the central nervous and the cardio vascular system. In addition, the caffeine

interference with the uptake and storage of calcium by the sarcoplasmic reticulum, it also increases the respiratory rate and cause bronchodilation and stimulate lypolgysis [30].

The stimulatory action of caffeine involves antagonism of adenosine receptors which are present in brain, blood vessels, kidneys, heart, the gastro intestinal tract and the respiratory hierarchy [31]. Adenosine is an adenine molecules attached to a ribose or deoxyribose sugar molecules. The similarity in chemical structure between the adenine portion of adenosine and the caffeine molecules is the key to how caffeine works [32]. Adenosine when bound to receptors of nerve cells, slow down nerve cell activity during sleep. Caffeine, being structurally similar to adenosine, has the potential to occupy adenosine receptors sites. When the caffeine molecules bind to the receptors doesn't cause the cells to slowdown; instead, the caffeine blocks the receptors and there by blocks the regulatory functions adenosine and produces the stimulatory effect [33].

2.3.4. Methods of Analysis

The GC-MS internal standard (1 mg/L) was added to the prepared samples prior to GC-MS analysis. Aliquots of 1 µL of the spiked samples were analyzed by GC-MS on an Agilent 5977GC/MSD GC-MS triple quad model (USA). The samples were injected in split less mode with the injector port temperature at 280 °C. A 30 m x 0.25 mm i.d. x 0.25 µm film thickness capillary column (Agilent, USA) was used. Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The column temperature program for the GC oven was as follows: initial temperature 70 °C, maintained for 2 min and then ramped at 20 °C to 230 °C, where it was held for 4 min [53]. Due to above mentioned facts many chemical and physical methods have been developed for the determination of caffeine in coffee and other beverages. The most widely used methods for the determination of caffeine in beverages are based on UV-Vis Spectrophotometry and partial least square [30], UV-Vis Spectrophotometry [34], derivative spectrophotometry, HPLC [21]. Fourier Transform Infrared (FTIR) Spectroscopy [35] NIR Reflectance Spectrometry [36], Raman Spectroscopy [37], Capillary Electrophoresis [38] .

The spectrophotometric method is fast, simple, accurate, reproducible and inexpensive procedure as compared to other methods, but it is not possible to determine caffeine directly in coffee beans by conventional UV-Vis absorption measurement due to the spectral overlap of UV absorbing

substances in the sample [19]. Derivative spectrophotometry is relatively easy, but; it is not reliable for the small concentration of caffeine in samples. By HPLC methods many caffeine contents were determined in various foods using different procedures since it provides the most reliable method.

2.3.5. Liquid-Liquid Extraction

Liquid-liquid extraction (LLE) is one of the most utilized techniques for sample preparation in various types of chemical analysis processes. Liquid–liquid extraction (LLE), also referred to as solvent extraction or partitioning, is a method for separating chemical entities based on their selective affinities for one of the phases in a two phase system, typically an aqueous and an organic phase. Due to its attractive characteristics such as versatility and scalability, LLE has been implemented in a variety of applications at industrial scale including metal extraction [38] and organic synthesis [39] in various sample pre-treatment processes (e.g., purification of biomolecules[40], ultra-sensitive measurement of analytes [41], pesticide analysis, and in analytical applications, e.g., for the determination of the lipophilicity during drug discovery and development [42].

The kinetics of liquid-liquid extraction of caffeine from aqueous solutions can be a function of both the chemical reactions taking place in the system and the rates of diffusion of the species present in the two phases [43]. The rate of solvent extraction can be characterized by the slow step of the overall reaction mechanism, which can occur either in the bulk (homogeneous reactions) or at the liquid-liquid interface or in a thin volume region very close to it [44]. In addition, diffusion processes can also be rate determining, as the chemical species have to be transferred from one phase to another for the reaction to occur, and the transport of material from the bulk to or from the interface can be significantly slower than the actual reaction.

3. MATERIALS AND METHODS

3.1.Sampling Area

Plant of Coffee beans was collected at the month of November 2021 from Mizan Teferi(town 568 km from Addis Ababa), Shebench (50 km), Debrework (28 km) and Sheko(19 km) far from town at the South-west region, Ethiopialatitude,6º 56.580'N, longitude, 35º 30.607'E.

a.

b.

Figure3. The map of Bench Maji zone(a) and coffee sample(b) (It's new name Bench Sheko zone)

3.2.Chemicals and apparatus

3.2.1. Chemicals

Analytical grade solvents and other chemicals employed in this study was chloroform (Aldrich-Sigma), acetone, methanol(98%ET), ethanol(95%ET),petroleum ether, 2% Hydrochloric acid, sulfuric acid, Sodium chloride, potassium iodide, Na_2SO_4all purchased from Sigma-aldrich (Milwaukee,WI,USA) KBr, Na_2CO_3, ferric chloride, ammonium hydroxide, ammonia, distilled water , coffee sample purchased from the local market (in Mizan, Sheko, DebreWork, Shebench) and standard caffeine.

3.2.2. Apparatus

Stand set up for liquid-liquid extraction Whatman filter paper No1&4, separatory funnel, rotary evaporator, volumetric flask, test tube, test tube holder, conical flask, autoclave, hot plate with magnetic stirrer, electrical balance (Mettler Toledo-AL204-IC America), inoculating loop, Sterilizer; Incubator, GC-MSD (Agilent 5977B USA) and refrigerator was used.

3.2.3. Sample collection

A 1 kg of coffee collected from each four different geographical location Mizan Teferi (town), Shebench (50 km far from town), Debrework (28 km) and Sheko (19 km) and packed in plastic bag.

3.3.Sample preparation

3.3.1. Roasting

The coffee beans first it was washed with tap water and roasting method was traditional method by stove at home for 15 minutes manually stirred until the color of coffee beans changed a red brown color with pop sound and cooled for 15 minutes.

3.3.2. Grinding

The roasted/raw coffee was grinded by traditional coffee grinder and sieved through 250µm sieve to get a uniform texture. All grinded samples were kept in polyethylene plastic bags until they were found to be analyzed.

3.4.Extraction

3.4.1. Extraction of caffeine for GC-MS analysis

A 1 g of roasted and raw powder coffee beans were separately added in to 500 mL beaker filled with 200 mL distilled water with magnetic stirrer. Then it was boiled at hot plate with magnetic stirrer for 40 minutes at 100 °C, the boiled mixture was cooled for 15 minutes then filter by Whatman filter paper No1, each 20mL of boiled coffee added in a 50 mL beaker then add 1:1 ratio of 20 mL chloroform and 20 mL each boiled roasted/raw coffee in each beaker and added the mixture in the separatory funnel by shaking (invert) up and down around 30 times and left for 10 minutes on stand then the foam eliminated. Lastly some sodium sulphate added on the filter paper to dehydrate and filtered from separatory funnel in to vial sealed by paraffin plastic and settled in refrigerator until GC-MS read [45].

3.4.2. Extraction of phytochemicals for screening tests

2 g of each raw and roasted coffee beans powder were taken separately and mixed to give 8 g of each raw and roasted sample. Each 8 g of raw and roasted samples were added into conical flasks and extracted sequentially with each 20 ml of petroleum ether, chloroform, acetone and methanol respectively, and then it was put in vial until the respective phytochemical test.

3.5.Determination of Caffeine by GC-MS spectroscopy

3.5.1. Standard preparation

The calibration curve was plotted in the optimized conditions of the procedure using solutions with caffeine concentration levels of 1 to 10 μg/L and was characterized with an R^2. The LOD based on 3σ/m, where σ and m are the standard deviation and slope of the calibration graph, Caffeine stock solution 1000 ppb was prepared by dissolving 15μg of pure caffeine in 1.00 mL of chloroform and prepared five working solutions of 1, 2, 3, 4, 5 and 10 μg /L[34].

3.5.2. Real sample preparation for GC-MS spectroscopy

15 μLeach extract roasted/raw sample added in 1.00mLchloroform in vial finally read the GC-MS that was already cooled [36].

3.6.Validation Method

Method validation is criteria used for evaluating analytical methods termed to validate in order to derive useful conclusions about the validity of the method. The following are method of validation

3.6.1. Accuracy

Recovery of the analytic spiked into sample matrix (measuring the analyte in sample matrix samples spiked with known analytical concentrations and determining the percentage).

Recovery (%) = $Xs-X/X\mathrm{add}$ x 100

Where, Xs = amount after spiked, X= amount before spiked and X add= the added solvent.

3.6.2. Limit of detection (LOD)

Determined by comparing the analytical signals at known low concentrations with those samples up to an analytical concentration that produces a signal equivalent to three times the standard deviation of the blank sample (3x σblank /S).

3.6.3. Limit of quantification (LOQ)

Standard deviation/slope ratio (LOQ = 10 σblank/S) where σ standard deviation and s slop.

3.6.4. Linearity and range

The linearity of a method measures how well a calibration plot of analytical response *vs.* Analyte concentration approximates a straight line or how well the data fit to the linear equation

Y = mx + c Where: *y* = analyte response/measured signal, *x* = analyte concentration of sample, *m* = slope of a line fit to the data (tangent), is a measure for the sensitivity of the procedure; the steeper the slope the more sensitive the procedure and *c* = intercept of a line fit to the data.

3.6.5. Data Analysis

All the experiments were done in duplicate and data were showed as mean ± standard deviation (SD). Results of caffeine in coffee by product sample analyses was compared against standards set by literature and the obtained data was analysis using simple descriptive assisted by ANOVA were used to compare mean concentration of caffeine.

3.7.Gas Chromatography-Mass Spectra Analysis

Natural caffeine in beverages was quantified by standard addition of caffeine external standard. The internal standard (1 mg/L) was added to the prepared samples prior to GC-MS analysis. Aliquots of 1 µL (Agilent Technologies 8890auto sampler) of the spiked samples were analyzed by GC-MS on an Agilent 5977B GC/MSD GC-MS triple quad model (USA). The samples were injected in split less mode with the injector port temperature at 280 °C. A 30 m x 0.25 mm i.d. x 0.25 µm film thickness, HP-5MS (5% phenyl, 95% methylpolysiloxane) capillary column (Agilent, USA) was used. Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The column temperature program for the GC oven was as follows: initial temperature 70 °C, maintained for 2 min and then ramped at 20 °C to 230 °C, where it was held for 4 min [46].

3.8.Phytochemical screening of coffee extract

The presence of the following phytochemicals was analyzed the procedures presented below.

Steroids (salkowiski test): 1 mL of the extract is dissolved in 10 mL of chloroform and equal volume of concentrated sulphuric acid is added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow color with green fluorescence indicating the presence of steroids [47].

Phenolic Compounds ($FeCl_3$ test): To 2mL of the extract, 5% ferric chloride solution is added. Deep blue black color indicates the presence of phenol [47].

Alkaloids (Wagner's test): 5 mL of 2% HCl was added to 2 mL of the extract in a test tube placed on a steam bath and warmed. It is filtered and divided into two parts for the following tests [48].

A few drops of Wagner's Reagent (iodine in potassium iodide) was added to one part of the filtrate in a test tube. A reddish brown precipitate is observed.

The formation of precipitation indicated the presence of alkaloids.

Flavonoids (alkaline reagent test): Extracts were treated with few drops of sodium hydroxide solution (0.1M). Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of Flavonoids [49].

Terpenoids(salkowiski test): Treating the extract in chloroform with few drops of concentrated sulphuric acid, shake well and allowed to stand for some time, formation of yellow colored lower layer indicate the presence of Terpenoids [50,51]

Anthraqunon test: (ammonia test)

The addition of ammonia to the extract show pink color if there is Anthraqunon

Saponins (foam test): Dilute the extract with distilled water and shake in graduated cylinder for 15 minutes. The formation of layer of foam indicates the presence of Saponins [52].

3.9.The bioassay of roasted and raw coffee extract

Antibacterial activities of solvent extracts were determined by disc diffusion method on nutrient agar medium [58] is used in order to check the antibacterial activity of sequential extract of each coffee extracts inhibitory activity were detected in terms of diameter of zones of inhibition (in mm) in comparison with standard antibiotic (gentamycin) as positive control and the solvent (10% aqueous DMSO) as a negative control. The antimicrobial activities of the crude extracts of each of the essential oils will be carried out by disc diffusion method: Muller Hinton agar are prepared according to the manufacturers recommendation by dissolving the required amount of the powder in distilled water and boiled to mix thoroughly and then, Muller Hinton Agar media, Petri dish, forceps, paper discs 6mm of diameter are sterilized by autoclave at 121°C for 15 min. The plates are taken to the laminar air flow hood and left until they are completely dried.

4. RESULTS AND DISCUSSION

4.1.Method Validation

4.1.1. Linearity

Standard caffeine solution (1 - 10 μg/L) was used to calibrate the GC-MS under an optimized condition and the obtained response was plotted against concentration (Figure 4). The linearity of the curve was good with a high value of deterioration coefficient (R^2 of 0.9945).

Table 1. Calibration data of standard caffeine

Concentration(μg/mL)	Peak Area
1	62269
2	152267
3	244666
4	359883
5	533264
10	1030392

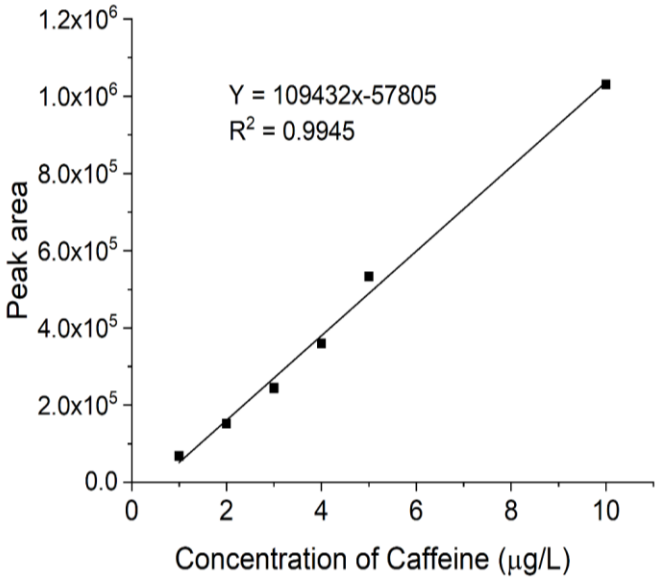

Figure 4. The graph of calibration

4.1.2. Accuracy

The accuracy of the caffeine extraction procedure was evaluated by determining the percent of recovery(%R) of the spiked raw and roasted coffee bean samples due to the lack of certified reference materials in the laboratory. Table 2 shows the %R for coffee bean samples. The obtained recoveries for the spiked raw coffee samples ranged from 100.6% to 117.6% and roasted coffee ranged from 83.77 to115.83%. These values are within the accepted range for the analysis of caffeine in coffee samples, indicating that the employed extraction procedure has the required accuracy for analysis

of the caffeine in coffee. The precision studies, expressed as RSD for both raw and roasted samples, were < 10%, which is also acceptable for the analysis of caffeine in coffee.

The LOD and LOQ were ranged from 0.48 – 1.07 and from 1.6 – 3.56 µg/L respectively.

Table 2. Recovery results of coffee bean samples

Coffee Source	**Raw spiked (µg/L)**	**Roasted spiked (µg /L)**	**Recovery percentage**			
			Raw %	**Spiked(µL)**	**Roasted %**	**Spiked(µL)**
Mizan	34.2	42.2	113.6	25	115.83	30
Debrework	32.35	34.95	106.8	25	89.17	30
Sheko	34.8	36.4	117.6	25	93.9	30
Shebench	30.1	37.9	100.64	25	99.6	30

4.2.The Caffeine Content of Coffee

The caffeine content of the four area Mizan, Debrework, Shebench and Sheko coffee. The graph of calibrationruns by GC-MS all are measured in duplicate. 15 µL of extract added to 1.00 mL of chloroform solvent in the vial and run to GC-MS.

Table 3. Concentration of caffeine in roasted and raw coffee beans extracts

Sample types	**Coffee samples**	**Weight (g)**	**Caffeine (µg/L)**
Roasted coffee	Mizan	1.00	7.45 ± 0.036
	Debrework	1.00	9.82 ± 0.0377
	Shebench	1.00	8.01 ± 0.071
	Sheko	1.00	8.22 ± 0.60
Raw coffee	Mizan	1.00	5.8 ± 0.32
	Debrework	1.00	5.62 ± 0.175
	Shebench	1.00	4.94 ± 0.39
	Sheko	1.00	5.42 ±0.175

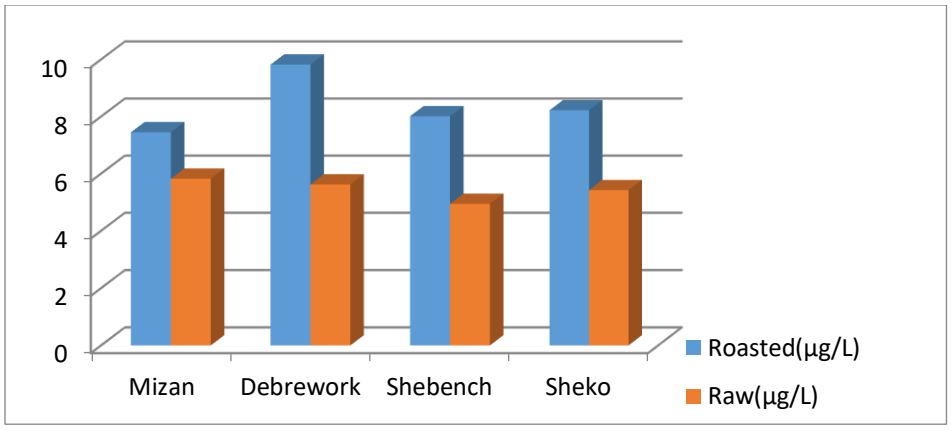

Figure 5. the caffeine level of roasted and raw coffee

4.3. Analysis with Other caffeine content

As the comparison shows, the proposed method is highly sensitive, requires a small amount of organic extraction solvent and with the detection limit that is lower than most of the mentioned methods. In the proposed method, there is a great surface area between the extraction solvent (chloroform) and aqueous sample phase, therefore, the preconcentration and isolation of caffeine is performance with a high efficiency.

In summary, all the coffee samples have the caffeine level in the range of the caffeine contents of different Arabica coffee [56]. The variation in caffeine level of green coffee beans samples may be due to geographical origins which might have different altitude, soil type, rain fall and other agricultural as well as environmental conditions.

Table 4. Comparison of green coffee caffeine content with the other country region by**(w/w)** % a, b, c and d represents the roasted coffee caffeine.

No	**Analysis method**	**extraction method**	**Place**	**Caffeine (w/w)%**	**Ref.**
1	HPLC	LLE	Wolyta	0.66	[56]
2	HPLC	LLE	Asendabo	0.84	[56]
3	HPLC	LLE	Silte	0.93	[56]
4	HPLC	LLE	Bonga	1.09	[56]
5	GC-MS	LLE	Mizan	0.74a	present work
6	GC-MS	LLE	Shebench	0.80b	present work
7	GC-MS	LLE	Sheko	0.82c	present work
8	GC-MS	LLE	Debrework	0.98d	present work

4.4. Phytochemical Screening of Coffee Extract

It was tested after measuring each 2 g of the four raw and roasted coffee sample of 8 g powder sequentially extracted by methanol, chloroform, acetone and petroleum ether as shown the photo at the appendix, alkaloid, phenol, flavonoid and terpenoids was found in coffee as shown in Table 5. The phytochemical screening test of roasted and raw coffee "+''represent presence and " –" represent the absence of photochemical.

Table 5. Phytochemical screening test

		Petroleum Ether		Chloroform		Acetone		Methanol	
No	**Testes**	**Raw**	**Roasted**	**Raw**	**Roasted**	**Raw**	**Roasted**	**Raw**	**Roasted**
1	Alkaloid	+	+	+	+	+	+	+	+
2	Phenol($FeCl_3$)	–	–	–	–	–	–	+	+
3	Flavonoid	–	–	–	–	–	–	+	+
4	Anthraqunon	–	–	–	–	–	–	–	–
5	Terpenoids	+	+	+	+	–	–	–	–
6	Saponins	–	–	–	–	–	–	–	–

4.4. The Bioassay of Roasted and Raw Coffee Beans

Inhibition zone of all extracts was zero mm that of gentamycin is 30 mm except for s.typi (29mm), inhibition zone of contrimazol for c.albicans was 16mm, that of DMSO was zero in all.

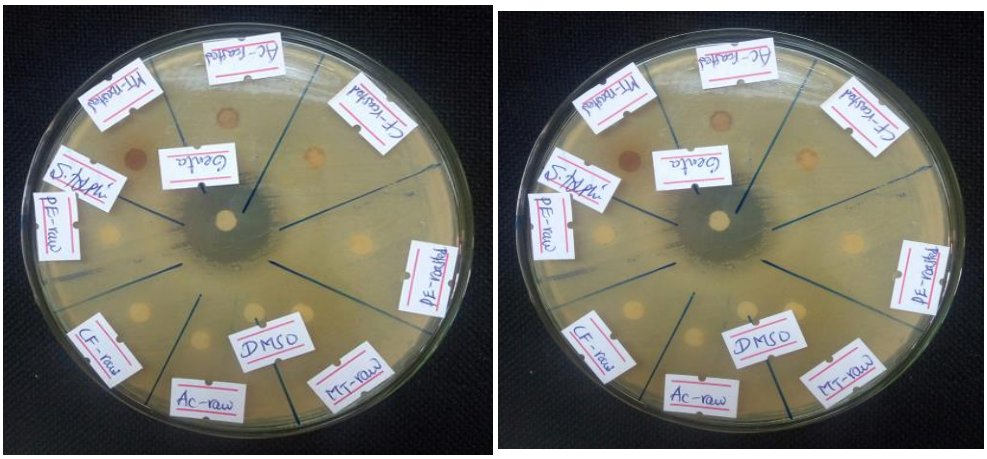

Figure 6. The Bioassay of raw and roasted coffee extracts

5. CONCLUSION AND RECOMMENDATION

5.0.Conclusion

Gas Chromatography - Mass spectroscopy method was employed for determination of level of caffeine in coffee samples of Bench Sheko zone and also the phytochemical screening test on roasted and raw coffee bean was studied. The results of this study showed that the caffeine contents of all raw coffee samples were higher than that of roasted coffee beans. The caffeine contents of roasted coffee beans ranged from 7.45 – 9.8*2* $\mu g/L$, whereas that of raw coffee beans was in the range of 4.94– 5.82 $\mu g/L$. Recovery (%) of rawcoffee caffeine was ranged from100.64% - 117.6%. And whereas that of roasted coffee beans was ranged from 93.9%-115.83%.

The study also showed that,the caffeine content of roasted coffee is higher than that of rawcoffee [59].This may be due to the increasing of caffeine during roasting.

5.1.Recommendation

Based on the result obtained in the present study the following points are recommended.

- The developed method is suitable for the determination of caffeine from coffee, so it is good using this method for production of caffeine.
- The various types of coffee beans in the community should be studied with regard to their caffeine content, so that one can know the amount of caffeine by extraction with environmentally familiar LLE solvent.
- It is better to do an advanced study on caffeine bulk production.
- Government and NGO body have to give attention on how caffeine extract management in efficient production and utility for other medicinal values.

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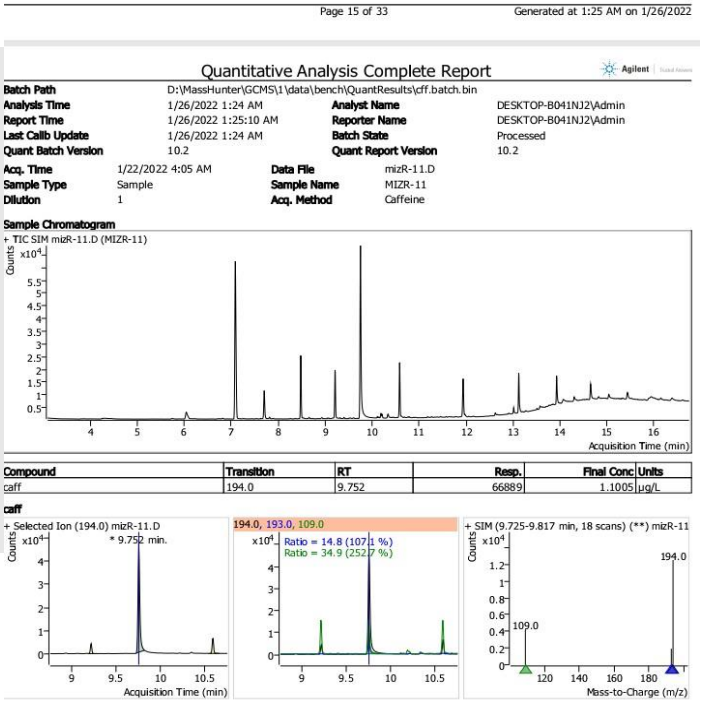
Appendix A.The read out of GC-MS of standard caffeineAppendix



Page 15 of 33 Generated at 1:25 AM on 1/26/2022

Quantitative Analysis Complete Report

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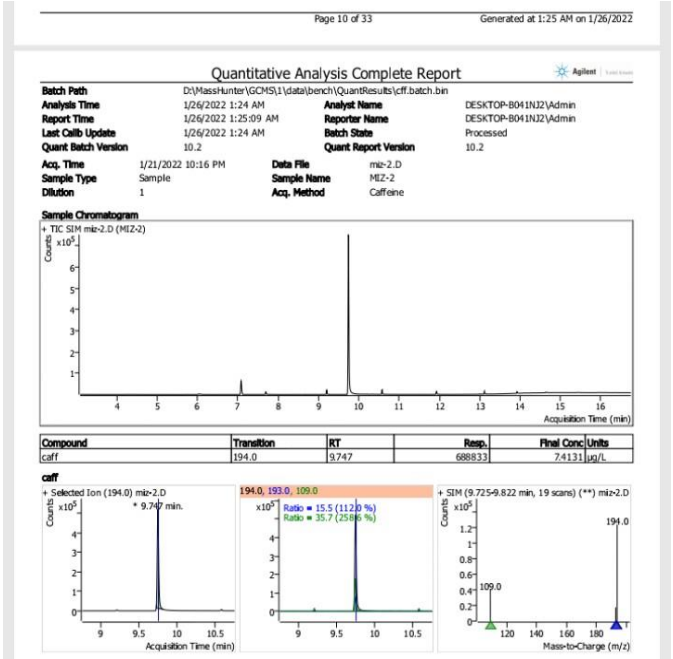
Sample Type	Sample	Sample Name	MIZR-11
Dilution	1	Acq. Method	Caffeine

Sample Chromatogram

Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.752	66889	1.1005	µg/L

caff



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Dilution	1	Acq. Method	Caffeine

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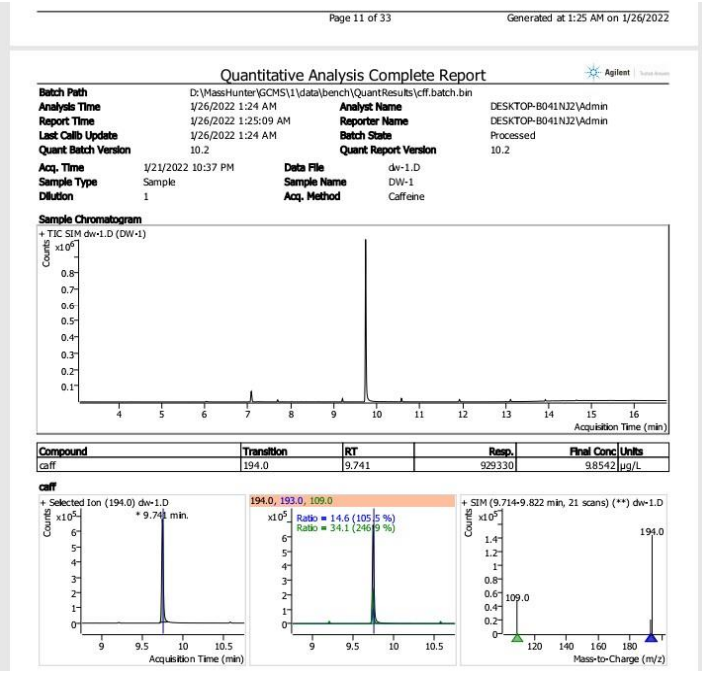

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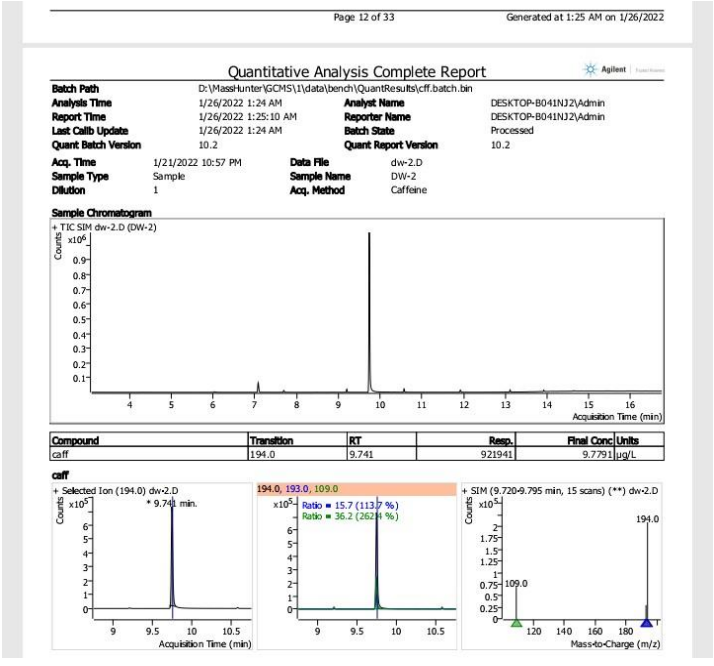
Sample Type	Sample	Sample Name	DW-1
Dilution	1	Acq. Method	Caffeine

Sample Chromatogram

Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.741	929330	9.8542	µg/L

caff



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Dilution	1	Acq. Method	Caffeine

Sample Chromatogram

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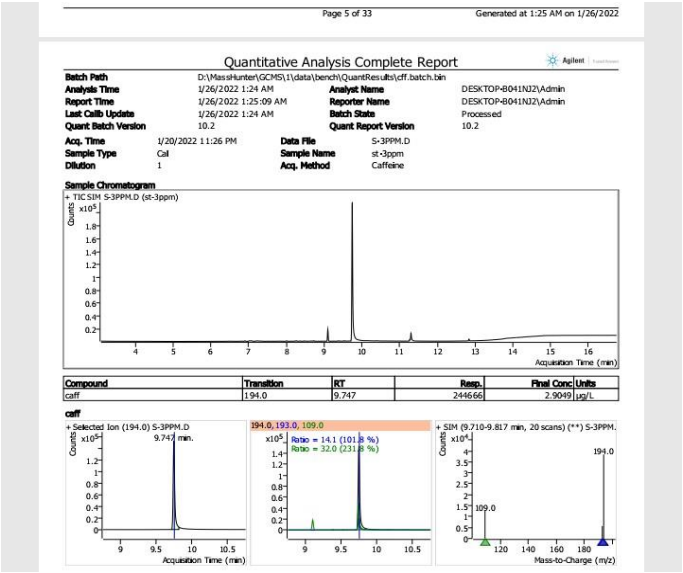
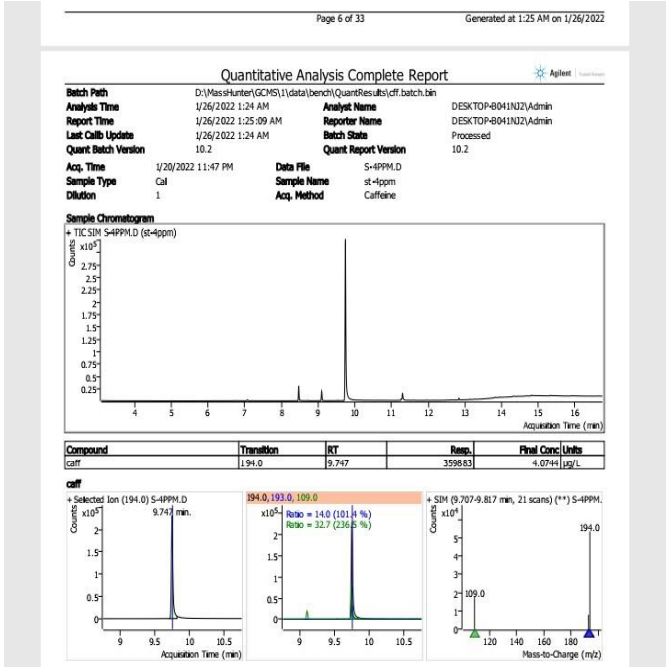
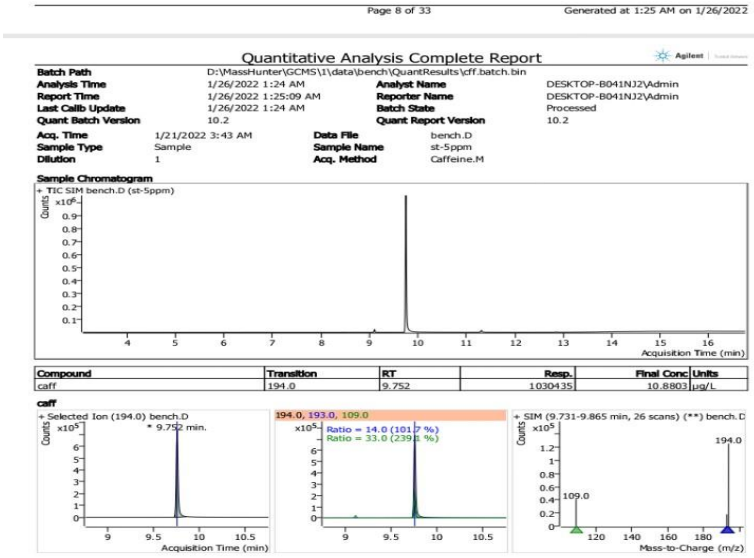
caff



B:



The Roasted GC-MS Read out



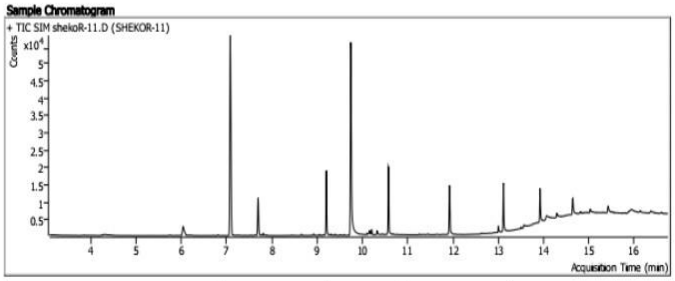
Appendix C: The Raw coffee GC-MS read out

Quantitative Analysis Complete Report

Agilent

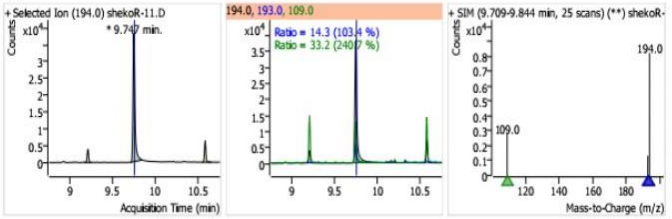
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/22/2022 6:08 AM	**Data File**	shekoR-11.D
Sample Type	Sample	**Sample Name**	SHEKOR-11
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram



Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.747	62367	1.0546	µg/L

caff

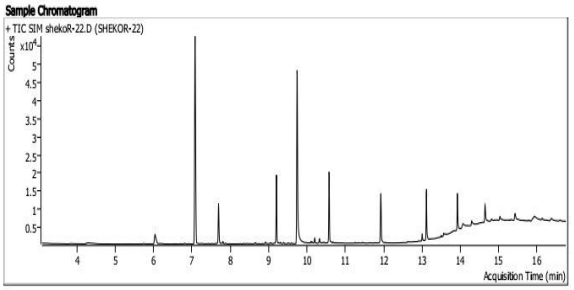


Quantitative Analysis Complete Report

Agilent

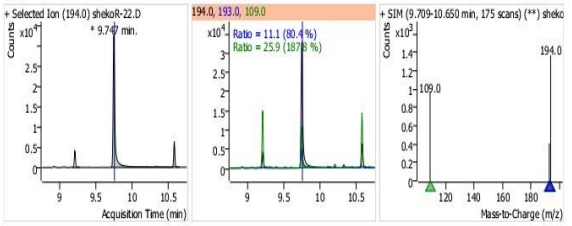
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/22/2022 6:28 AM	**Data File**	shekoR-22.D
Sample Type	Sample	**Sample Name**	SHEKOR-22
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram



Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.747	68455	1.1164	µg/L

caff



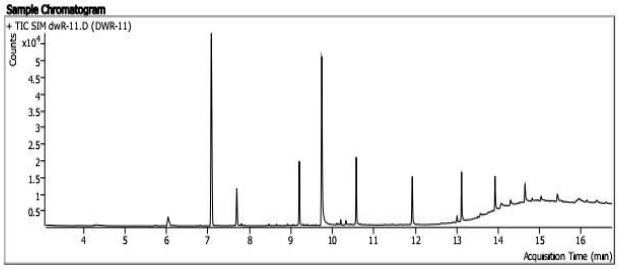
5/2022

Quantitative Analysis Complete Report

Agilent

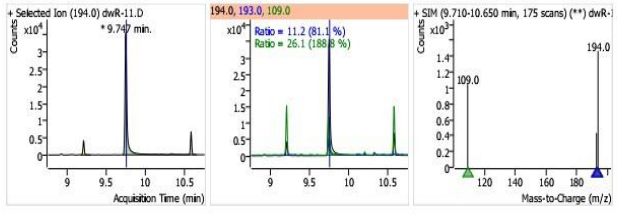
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/22/2022 4:46 AM	**Data File**	dwR-11.D
Sample Type	Sample	**Sample Name**	DWR-11
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram



Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.747	73130	1.1639	µg/L

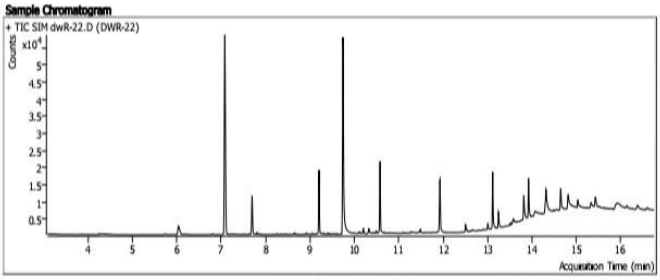
caff



Quantitative Analysis Complete Report

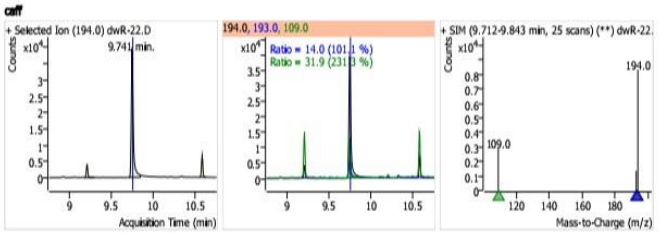
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/22/2022 5:06 AM	**Data File**	dwR-22.D
Sample Type	Sample	**Sample Name**	DWR-22
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram



Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.741	65530	1.0867	µg/L

caff

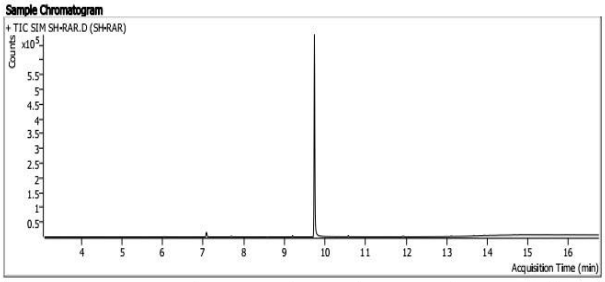


Quantitative Analysis Complete Report

Agilent

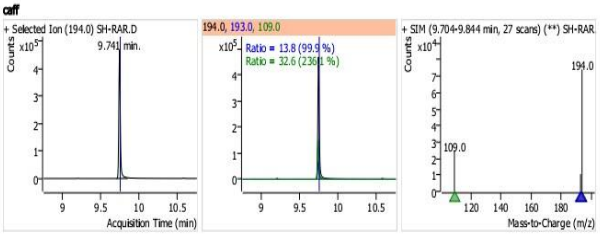
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/24/2022 12:41 AM	**Data File**	SH-RAR.D
Sample Type	Sample	**Sample Name**	SH-RAR
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram



Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.741	644285	6.9610	µg/L

caff

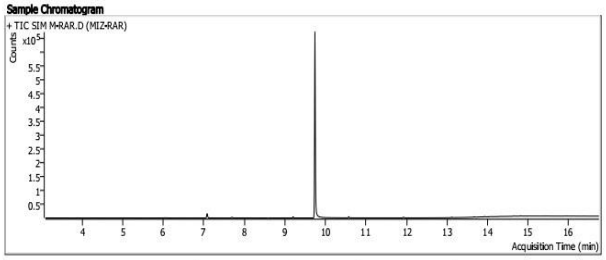


Quantitative Analysis Complete Report

Agilent

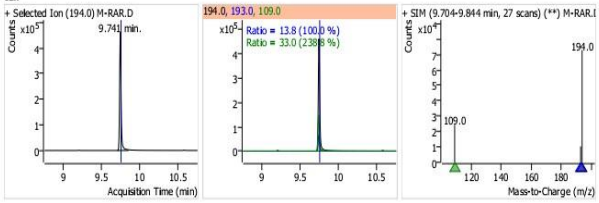
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/24/2022 1:01 AM	**Data File**	M-RAR.D
Sample Type	Sample	**Sample Name**	MIZ-RAR
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram



Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.741	632614	6.8425	µg/L

caff

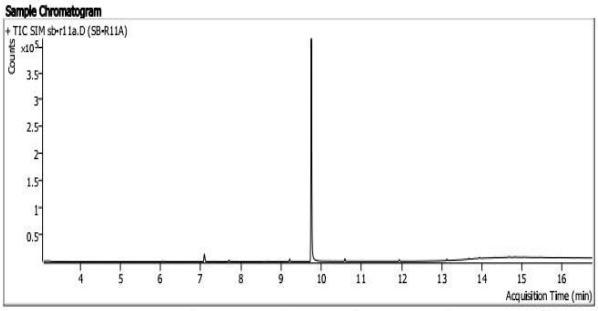


Quantitative Analysis Complete Report

Agilent

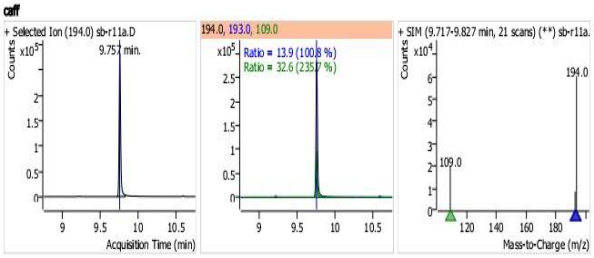
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/25/2022 10:41 PM	**Data File**	sb-r11a.D
Sample Type	Sample	**Sample Name**	SB-R11A
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram



Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.757	406107	4.5435	µg/L

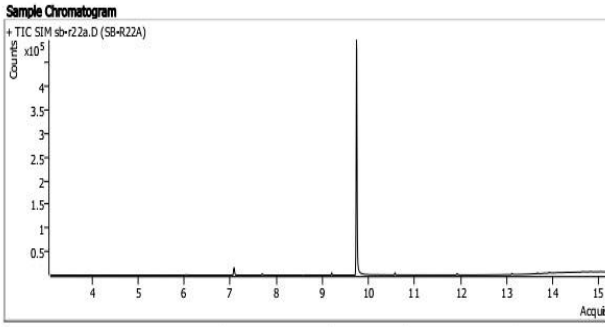
caff



Quantitative Analysis Complete Report

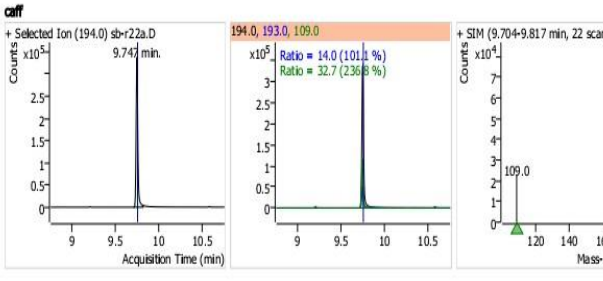
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	**Analyst Name**	DESKTOP-B041NJ2\Adm
Report Time	1/26/2022 1:25:10 AM	**Reporter Name**	DESKTOP-B041NJ2\Adm
Last Calib Update	1/26/2022 1:24 AM	**Batch State**	Processed
Quant Batch Version	10.2	**Quant Report Version**	10.2
Acq. Time	1/25/2022 11:01 PM	**Data File**	sb-r22a.D
Sample Type	Sample	**Sample Name**	SB-R22A
Dilution	1	**Acq. Method**	Caffeine

Sample Chromatogram

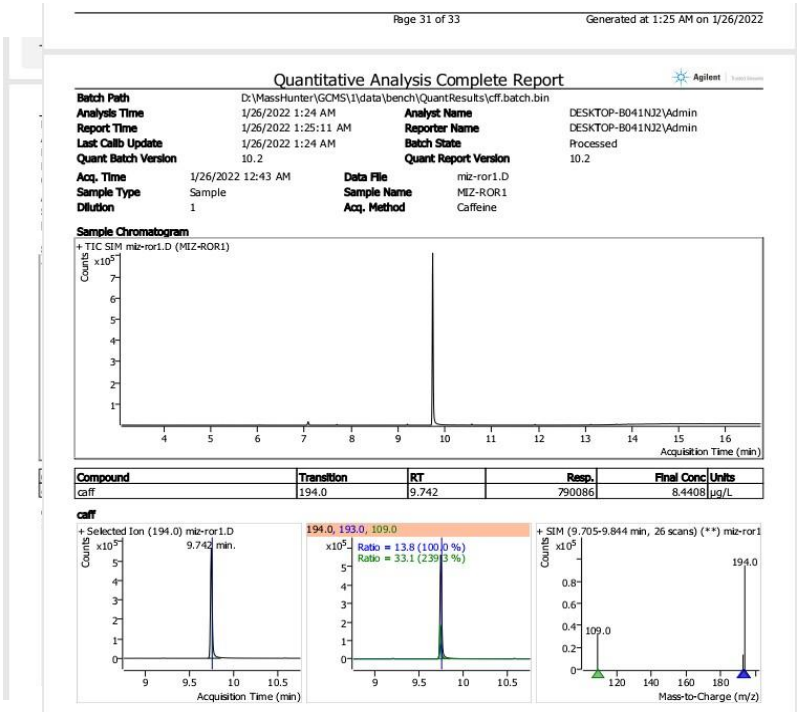


Compound	Transition	RT	Resp.	Final C
caff	194.0	9.747	483899	5.3

caff



Appendix D: Recovery read out of GC-Ms



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Quantitative Analysis Complete Report

Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	Analyst Name	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:11 AM	Reporter Name	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	Batch State	Processed
Quant Batch Version	10.2	Quant Report Version	10.2
Acq. Time	1/26/2022 12:43 AM	Data File	miz-ror1.D
Sample Type	Sample	Sample Name	MIZ-ROR1
Dilution	1	Acq. Method	Caffeine

Sample Chromatogram

Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.742	790086	8.4408	µg/L

caff

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Quantitative Analysis Complete Report

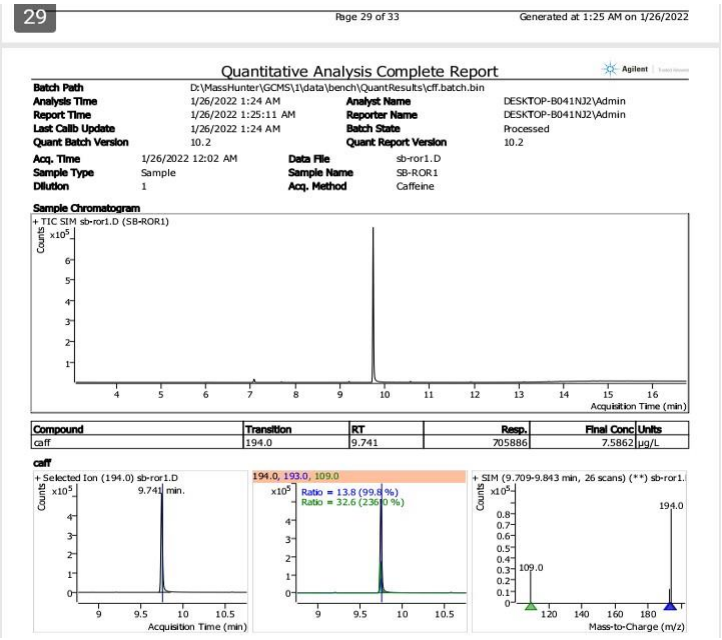
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	Analyst Name	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:10 AM	Reporter Name	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	Batch State	Processed
Quant Batch Version	10.2	Quant Report Version	10.2
Acq. Time	1/25/2022 11:42 PM	Data File	sheko-rd.D
Sample Type	Sample	Sample Name	SHEKO-RD
Dilution	1	Acq. Method	Caffeine

Sample Chromatogram

Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.741	708969	7.6175	µg/L

caff

29



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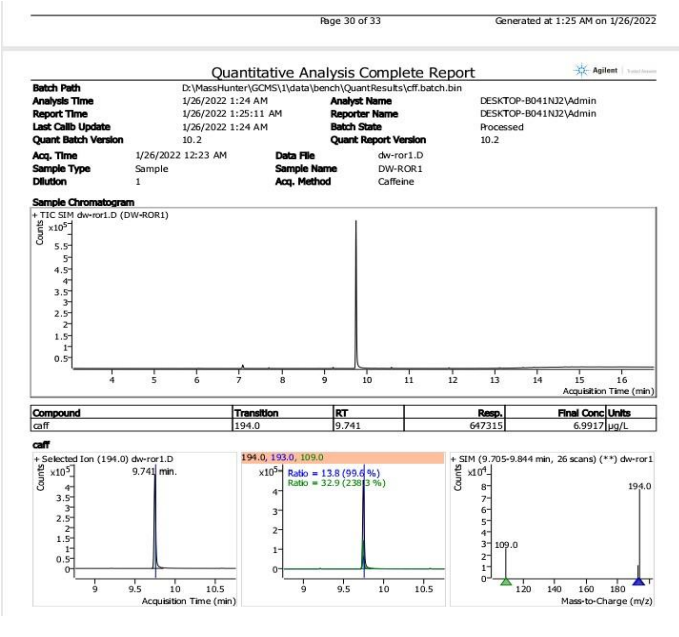
Quantitative Analysis Complete Report

Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	Analyst Name	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:11 AM	Reporter Name	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	Batch State	Processed
Quant Batch Version	10.2	Quant Report Version	10.2
Acq. Time	1/26/2022 12:02 AM	Data File	sb-ror1.D
Sample Type	Sample	Sample Name	SB-ROR1
Dilution	1	Acq. Method	Caffeine

Sample Chromatogram

Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.741	705886	7.5862	µg/L

caff



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Quantitative Analysis Complete Report

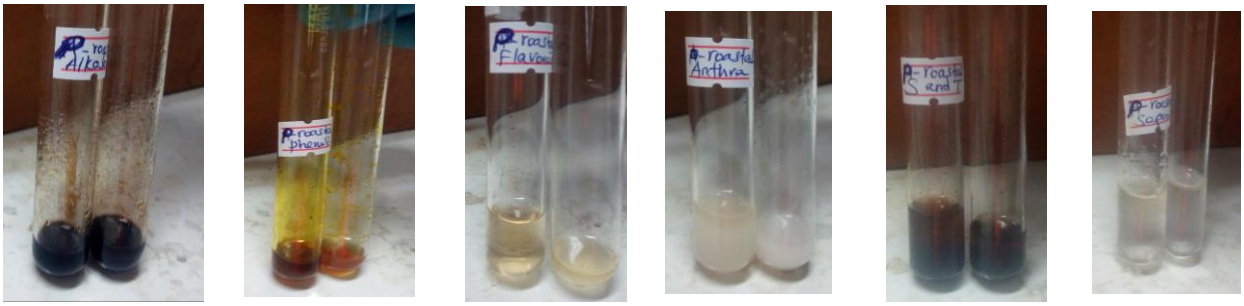
Batch Path	D:\MassHunter\GCMS\1\data\bench\QuantResults\cff.batch.bin		
Analysis Time	1/26/2022 1:24 AM	Analyst Name	DESKTOP-B041NJ2\Admin
Report Time	1/26/2022 1:25:11 AM	Reporter Name	DESKTOP-B041NJ2\Admin
Last Calib Update	1/26/2022 1:24 AM	Batch State	Processed
Quant Batch Version	10.2	Quant Report Version	10.2
Acq. Time	1/26/2022 12:23 AM	Data File	dw-ror1.D
Sample Type	Sample	Sample Name	DW-ROR1
Dilution	1	Acq. Method	Caffeine

Sample Chromatogram

Compound	Transition	RT	Resp.	Final Conc	Units
caff	194.0	9.741	647315	6.9917	µg/L

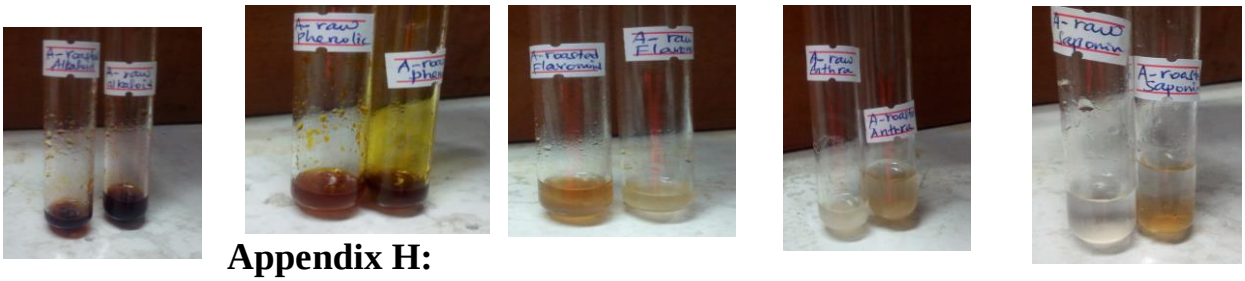
caff

Appendix E: The photo of petroleum ether extraction (p=petroleum ether)



Appendix F: photo of chloroform extraction(C= Chloroform)

Appendix G: photo of Acetone extraction (A=Acetone)



Appendix H:
Photo of Methanol extract (M= Methanol)