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M. Sc THESIS ON

DEVELOPMENT OF SALTING OUT ASSISTED LIQUID-LIQUID EXTRACTION METHOD FOLLOWED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF CAFFEINE AND NICOTINIC ACID IN RAW AND ROASTED COFFEE

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BY

GADISA CHIRFA MOSISA

ADVISORS

ABERA GURE (PhD)

YARED MERDASSA (PhD)

A Thesis Submitted to School of Graduate Studies, College of Natural Sciences at Jimma University in partial Fulfillment of the Requirements for the Degree of Master of Science in chemistry (Analytical)

Advisors **Signature** **Date**

1. Abera Gure (PhD) _____ _____
Academic Rank: Assistant Professor
Department of Chemistry
College of Natural Sciences
Jimma University

2.Yared Merdassa (PhD) _____ _____
Academic Rank: Assistant Professor
Department of Chemistry
College of Natural Sciences
Jimma University

Jimma University

School of Graduate studies

Development of Salting out Assisted Liquid-Liquid Extraction Method Followed by High Performance Liquid Chromatography for the Determination of Caffeine and Nicotinic Acid in Raw and Roasted Coffee

By

Gadisa Chirfa Mosisa

A Thesis Submitted to School of Graduate Studies Jimma University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry (Analytical)

Approved by Board of Examiners

External Examiner	Signature	Date
_____	_____	_____
Internal Examiner		
_____	_____	_____

Advisors

1. Abera Gure (PhD)	_____	_____
2. Yared Merdassa (PhD)	_____	_____
Head Department, Chemistry		
Tsegaye Deyou (PhD)	_____	_____

DECLARATION

Here the undersigned, declare that this thesis is my original work and has not been presented yet for research in any other universities.

Gadisa Chirfa

_____

This M.Sc Thesis has been submitted with our approval as supervisors:

Abera Gure (PhD)

_____

Yared Merdassa (PhD)

_____

Chemistry Department

Jimma University, Ethiopia

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List of abbreviations

ECX	Ethiopian Commodity Exchange
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography Diode Array Detector
HPLC-MAE	High Performance Liquid Chromatography Microwave assisted Extraction
HPLC-UV	High Performance Liquid Chromatography Ultra violet
HPLC-VWD	High Performance Liquid Chromatography Variable Wave Detector
IP	Ion Pair
LC-MS	Liquid Chromatography Mass Spectroscopy
LL	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
ppm	Parts per millions
RP-HPLC-UV	Reversed Phase High Performance Liquid Chromatography Ultra violet
RR	Relative Recovery
rpm	rate per minute
RSD	Relative Standard Deviation
SALLE	Salting out Assisted Liquid-Liquid Extraction
TBAHS	Tetra Butyl Ammonium Hydrogen Sulfate

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Abstract

In this study, salting out assisted liquid-liquid extraction (SALLE) method coupled with high performance liquid chromatography (HPLC) has been proposed for determination of caffeine and nicotinic acid from raw and roasted coffee samples. In the study, various parameters affecting chromatographic separations including mobile phase composition, injection volume, flow rate, wavelength, and column temperature as well as experimental parameters affecting the extraction efficiency of SALLE method such as type and volume of organic solvent, type and amount of salt, pH, concentration of ion pair reagent tetrabutylammonium hydrogen sulfate (TBAHS) were studied and optimum conditions were established. The calibration curves which were constructed by spiking ultrapure water at five different concentration levels and extracting with the optimum SALLE conditions exhibited wide linearity, with coefficient of determination R^2, 0.9953 and 0.9999 for nicotinic acid and caffeine respectively. The limits of detection (LODs) of the developed method were 0.2 and 0.8 $mgkg^{-1}$ for nicotinic acid and caffeine respectively. Intra- and inter-day precision studies were also performed at two concentration levels and demonstrated satisfactory precision, which is less than 10% RSD in both studies. Relative recovery studies were also ranging from 82-122% for both raw and roasted coffee samples. Hence, from the observed results the developed method is effective and could be utilized as attractive alternative for the determination of caffeine and nicotinic acid in raw and roasted coffee samples.

1. Introduction

1.1 Background of the study

Coffee is botanically belongs, to the *Rubiaceae* family, genus *Coffea*. The word "coffee" originated from the name of a region in Ethiopia where coffee was first discovered 'Kaffa' [1]. The major coffee growers of the country are Oromia Regional State, and Southern Nations and Nationalities People region. Some of the varieties of the coffees in the country are based up on the area of their production area and are classified as Harar, Jimma, Limmu, Wollega (includes Nekemte and Gimbi), Gedeo, Yirgacheffe, Sidamo, and Kaffa coffees [2, 3]. Although around 500 coffee species have been identified worldwide, only two are economically important. *Coffea arabica*, also known as C*offee arabica*, is responsible for approximately 70% of the global market, and *Coffea canephora* or C*offee robusta* (commercial name of one of the main *C. canephora* cultivars) accounts for the rest [4].

Most of coffee produced and consumed largely including Ethiopia is *Coffee arabica* [5]. In the production process there are different environmental conditions which affects its quality (location, altitude, weather conditions, soil and fertilization composition, cultivation, harvesting of the beans, and drying methods) are some of the factors that affect this tiny coffee bean changing chemical composition [6]. Its berries and their seeds undergo several processes from land of production to a marvelous ceremony indigenous to Ethiopia. From southwestern region of the country Jimma zone encompasses the largest share in production of *Coffee arabica* [7].

Many factors influence the overall quality and composition of the raw coffee bean. The cup quality is a highly complex trait, and depends on physical and sensory qualities with the variety from plant growth to cup test [8, 9]. Tools to identify its qualities such as color, shape, size, and the defects of green coffee beans are classified using eye. But, it is difficult to single out among coffee varieties after roasting and grounding. The roasting of coffee beans is another very important step in its processing, since specific organoleptic properties (flavors, aromas, and color) are developed and affect its quality and as a consequence the excellence of its beverage [10, 11]. During roasting, depending on the roasting time and temperature, highly complex process, i.e., hundreds of chemical reactions and changes occur simultaneously. These changes include the Maillard and Strecker reactions, degradation of polysaccharides, proteins, trigonelline, and chlorogenic acids [12]. Coffee is the world's most popular beverage after water, and one of the most important raw materials with in

the international trade for which quality is typical [13]. Studies indicated that the composition of hydrosoluble compounds, such as trigonelline, caffeine, chlorogenic acid, and nicotinic acid could be used as indicators for differentiation of coffee species [14].

As it has been presented in Furihata et al. [15], different analytical techniques such as gas chromatography (GC), high-performance liquid chromatography (HPLC), isotope-ratio mass spectrometry (IRMS), visible micro-Raman spectroscopy, ultraviolet visible absorption spectroscopy (UV-vis), and liquid chromatography-mass spectrometry (LC-MS), have been utilized to determine the chemical composition of coffee.

Sample preparation is the process of converting a sample into to suitable or concentrate target analytes for analysis. It affects efficiency, reproducibility and robust of the method [16]. There are several traditional and modern sample preparation techniques used in simultaneous determination of caffeine and nicotinic acid using chromatography, which includes solid-liquid extraction, solid-phase extraction, liquid-liquid extraction, microwave-assisted extraction (MAE), ultrasound-assisted extraction, solid-phase micro-extraction (SPME), supercritical-fluid extraction (SFE), and others has been used [17].

Salting out assisted liquid-liquid extraction (SALLE) is a sample pretreatment technique in which the appropriate amount of salt is added to a mixture of aqueous sample and water-miscible organic solvent in order to cause phase separation [18]. It involves a simple one-step solvent extraction of analytes from the sample followed by salting out the water-miscible organic solvent by adding inorganic salt, like ammonium sulfate, sodium chloride, calcium chloride, potassium carbonate, calcium sulfate or magnesium sulfate [19]. The method is a rapid, simple, cheap, convenient and environmentally safe sample pretreatment method in which the obtained extracts could be evaporated and reconstituted in a suitable solvent or directly injected into HPLC, capillary electrophoresis (CE) or GC instruments [18, 19]. SALLE is usually sued for extraction of relatively polar compounds and thus, it utilizes water miscible organic solvents such as methanol, ethanol, 1-propanol, acetonitrile, acetone and ethyl acetate as an extraction solvent. [20].

In the separation of charged analytes using chromatography, careful adjustment of the mobile phase pH, which results in a non-ionized analytes or ion suppression, and or use of ion-pairing agent as a mobile phase is crucial [21]. The ion-pairing forms ion paired association with the analyte ions and thus, could be separated by reverse phase mode [21].

The chemical composition in coffee (water soluble and water insoluble) contributing to good flavor and specialty grade needs the application of analytical laboratory to shift the traditional cup test discriminators of trained panels to modern way of quality control or cup quality determination [22]. Even though numerous techniques were recognized in other parts of the world, there were no systematic way of discriminating chemical components of coffee by SALLE for simultaneous determination of caffeine and nicotinic acid. Therefore, this study was mainly focuses on method development of SALLE followed by HPLC-UV detector for determination of caffeine and nicotinic acid in raw and roasted coffee.

1.2 Statement of the problem

Coffee is one of the most popular and widely consumed beverages throughout the world due to its pleasant taste, aroma and its stimulant effect. Its quality depends on the way it is produced, processed, and chemical composition. The visual way of identifying a good (shape, color, and size) is a difficult after it is roasted and ground releasing excellent flavor. Therefore, analytical tools are indispensable method in discriminating the chemical composition in varieties of coffee produced around the world. These methods were practiced for long time in coffee producing countries to grade and differentiate a cup quality coffee provided for world market. In Ethiopia, still traditional ways of cuppers taste were used to identify quality, in grading of the sample to verify the actual characteristics of the coffee. Among the many chemical composition of coffee caffeine, trigonelline, chlorogenic acids, and nicotinic acids are fabulous water soluble markers of coffee quality. Thus, this study focuses on analytical method development of salting out assisted liquid-liquid extraction followed by HPLC for determination of caffeine and nicotinic acid in coffee.

1.3 Objectives of the study

1.3.1 General objectives

- To develop a salting out assisted liquid-liquid extraction for the determination of caffeine and nicotinic acid from coffee using high performance liquid chromatography.

1.3.2 Specific objectives

- To optimize chromatographic conditions (HPLC) for the determination of caffeine and nicotinic acids in coffee.
- To optimize experimental parameters for determination of caffeine and nicotinic acid using salting out assisted liquid-liquid extraction.
- To quantify the amount of caffeine and nicotinic acids in raw and roasted coffee.
- To compare the composition of coffee discriminators in raw and roasted coffee.

1.4 Significance of the study

Coffee cupping is a technique used by panels to evaluate the flavor profile of its quality, to understand minor differences between growing regions, to evaluate coffee for consistence and defects to subsequently make buying decision, often referred as drinking quality or liquor quality, is an important attribute of its grading and acts as yardstick for price determination. But it is biased due to personal subjectivity or semi-objectivity and it is a traditional approach. So, development of effective and reliable analytical method in determination of chemical composition of coffee helps to replace the traditional approach and thus, shifts to the modern techniques in grading and determination of quality markers. Therefore, the findings of this thesis work would:

- help to determine coffee composition (caffeine and nicotinic acid) from raw and roasted coffee that contribute to its quality.
- provide an important base line information in identification of coffee quality markers.
- used as a guide line for further study in similar work.

2. Review of Related Literature

2.1 Coffee History

Coffee grown worldwide can trace its story back centuries to the ancient coffee forests of Ethiopian in Oromia region, goat herder, Kaldi, noticed that after eating berries from a certain tree, his goats became so energetic and unusually agitated that they did not want to sleep at night. According to this legend the potential of beloved bean was discovered for the first in this manner [23].

This plant is indigenous to the former Kaffa region in Ethiopia discovered some 2,000 years ago [24]. It belongs to the genus *Coffea*, in the *Rubiaceae* family. There are about 500 species of genus *Coffea*, but only two of these species are commercially explored worldwide [25]. *Coffea arabica* is considered as the noblest of all this plants and providing more than 70% of world's production [26]. It is mainly produced in the Southwest (former Kaffa, Ilu Abba Bora and Wollega), South East (Bale and Arsi), South (former Sidamo), and South East (Harar) parts of the country. In these areas the famous coffee types are known universally and exported by the names Limmu, Gimbi, Yirgacheffe, Harar, etc. of top price [27].

Ethiopia is the center of origin for *Coffea arabica* and produces a range of typical and has ample potential to sell a large number of specialty coffees [28]. Its production in the country is almost exclusively situated in the two regions, Oromia and the Southern Nations, Nationalities, and People Regions in the South and West of the country [29]. Moreover, there are wide ranges of variability among its types in each regions and woredas due to climate, altitude, temperature, rainfall, soil type, and pH will have a great influence on its characteristics from its chemical content: flavor and aroma [30, 31]. Thus, its quality varies from one region to another and even from one particular farmland to another that exists in the same woredas [31].

Coffee is one of the most agricultural products in the international trade [32]. It is the second commercialized commodity next to petroleum [32, 33]. Moreover, it is the commodity of great economic, social and environmental importance to coffee cultivating countries, particularly for developing countries [34]. Ethiopia accounts for about 3% of the global coffee market and about 60% of its foreign income. Besides, almost half of the country annual production is consumed domestically [32, 35]. It can be processed as sun-dried (natural), and wet (washed). This indicates that, processing to maintain the inherent quality and subsequent value of coffee [35].

The complexity of composition and variability of its aroma, taste, and health properties are affected by various factors (composition and origin, roasting and brewing process) [36]. The aroma of green seeds are quite different from what we imagine when we hear the word coffee. It is only through roasting that the characteristic aroma and flavor of coffee is developed [37].

From its chemistry point of view, roasting is a complex process since hundreds of chemical reactions take place simultaneously. Some examples include Maillard and Strecker reactions degradation of proteins, polysaccharides, trigonelline and chlorogenic acids. Sugars and trigonelline will act as aroma precursors, originating several substances (furans, pyrazines, pirroles, pyridines, etc.) that will affect both the flavor and aroma of the beverage [38]. Appraisal of trigonelline, chlorogenic acids and caffeine, in both green and roasted coffee, could be of relevance in launching its quality [39].

The quality of coffee used for beverage is related to the chemical composition of the roasted beans, which, in turn, is affected by post-harvest processing conditions (drying, storage, roasting and grinding) [40]. Its roasting process transforms into the distinctively aromatic, flavorful, brittle beans that we recognize as coffee usually heating at 200–240 ℃ for 10–15 min depending on the degree of roasting required, which is generally evaluated by colour (light, medium and dark) [39-40].

2.2 Chemical Compositions of Coffee

Many components remain undetected and little is known about the majority of the substances present in the coffee bean that we drink [41]. Non-volatile compounds found in green beans are chlorogenic acids, caffeine, trigonelline, soluble fiber, and diterpenes and the volatiles one includes volatile alcohols, esters, hydrocarbons, and aldehydes, Ketones, pyrazines, furans, and sulfur are some among the identified ones. Among the numerous compositions found in coffee (caffeine, trigonelline, nicotinic and Chlorogenic acids) are water soluble and (kahweol and casfestol) are fat-soluble [42 - 44].

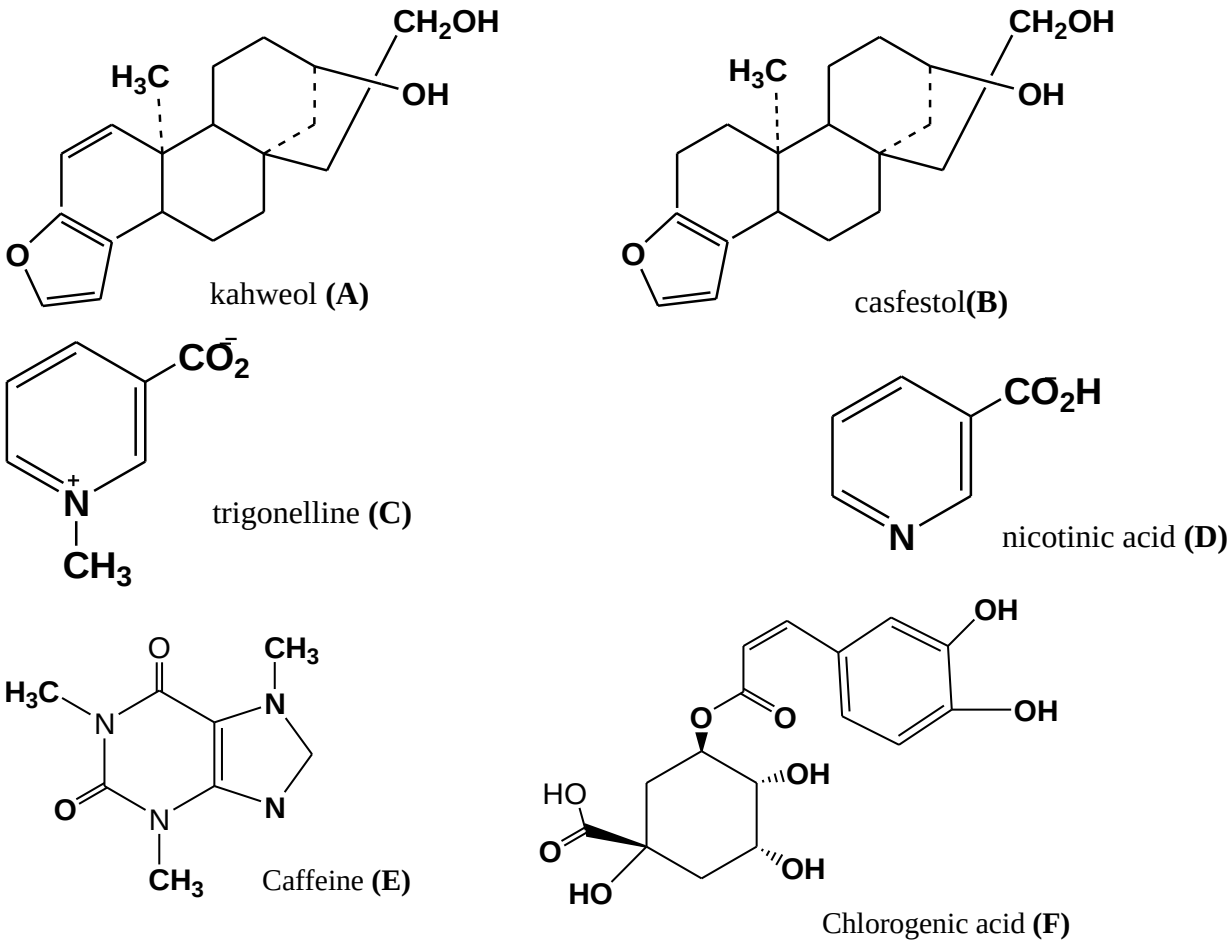

Figure 2.1 Chemical Structure of some Organic Compounds in Coffee [45]

These chemical compositions of raw and roasted coffee beans are assumed to be the main factors that control beverages quality [46]. Compound **E** is the major alkaloid present in green beans and its content is correlated with the quality of the beverage, since it contributes to the brew bitterness [47]. Compound **C** contents are almost 50% degraded during roasting, with the formation of other compounds, namely nicotinic acid, pyridine, 3-methyl-pyridine, and methyl ester of nicotinic acid [48]. Its composition depends on the genotypes and geographic area of origin, as well as of cultural practices, maturation, and post-harvest conditions, particularly storage [48].

2.2.1 Caffeine

Caffeine is a naturally occurring alkaloid found in tea leaves, coffee beans, kola nuts, cocoa beans and other plants [49]. It is chemically known as Trimethylxanthine, white crystalline solid powder, and has a bitter taste [50]. It causes various physiological effects such as relaxation of bronchial

muscle, stimulation of the central nervous system, gastric acid secretion, and dieresis and also used as a flavoring agent in a variety of beverages, including some soft drinks and energy drinks. On the other hand, analysis of caffeine content in coffee beans and tea leaves are also used as an additional tool for evaluating coffee and tea quality [49-50].

2.2.2 Nicotinic Acid

Nicotinic acid is a colorless crystalline substance slightly soluble in water and very stable in dry form. It is a form of vitamin B_3 which can be found naturally in meat, eggs and dairy products also found in coffee in which its deficiency result in pellagra that is characterized by diarrhea, dermatitis, dementia and death [51].

Table 2.2.1 physicochemical parameters of caffeine and nicotinic acid

Parameters	Caffeine	Nicotinic acid
Melting point (°C)	238	237
Boiling point (°C)	178	260
Acidity (pK_a)	10.4	2.0,4.85
water solubility (g L^{-1})	2.17	18
Density (gcm^{-3})	1.2	1.473
Molecular formula	$C_8H_{10}N_4O_2$	$C_6NH_5O_2$
Molar mass ($gmol^{-1}$)	194.19	123.11
Appearance	Odorless, white needles or powder	White, translucent crystals
pH	6.9	2.7

2.3 Roasting

In order to obtain a good quality cup of coffee with specific organoleptic properties (flavor, aroma and color), the step of roasting is very important. Its characteristics flavor and aroma results from a combination of hundreds of chemical compounds produced by the reactions that occur during the process [52-53]. The process is carried out under typically controlled by temperature, time, weight loss, and color parameters. The most common way to describe the roasting levels is by the color of

the roasted beans, ranging from light to dark or extra dark [54]. The quality of beverage is closely related to the chemical composition of the roasted beans [55-56].

Compound **D** is formed by the pyrolysis of Compound **C**. During heating its component at 210°C was rapidly reduced and **D** is formed in an inverse proportion. The maximum amount of **D** is formed after heating for 20 min, and almost all **C** disappeared after heating for 60 min [57].

Compound **E** is extracted from the seed of the coffee plant and the leaves of the tea bush in infusions and also found in various kinds of foods and drinks that we consume in our daily life. It acts as a central nervous system stimulant, temporarily warding off drowsiness and restoring alertness [58]. The content of **E** is increased with temperature of roasting depending on the degree of roasting [58].

2.4 Methods of Determination of Caffeine and Nicotinic Acid

Several analytical techniques have been used in the determination of chemical composition in coffee; HPLC, high performance gel filtration chromatography (HPGF, HPLC/MS, GC, and CE, in combination with various detectors, such as UV/Vis detector, diode array detector (DAD), refractive index (RI) detector, and chemiluminescence detector (CL) [59-60].

The more recent methods of simultaneous determination of caffeine and nicotinic acid are based on the reversed phase high performance liquid chromatography (RP-HPLC) combined with several detection methods like UV, mass spectrometer (MS), infrared (IR) and Nuclear magnetic resonance (NMR) spectroscopy [61-62]. RP-HPLC is accurate, sensitive, and cost-effective for simultaneous determination of nicotinic acid and water soluble vitamins [62].

2.5 Liquid-Liquid Extraction

Liquid -liquid extraction is one of a sample pretreatment method that have been utilizing aqueous of two-phase systems. The systems have been favored due to non-toxic and environmentally safe for the separation processes in method development [63].

The salt effect may be used for removing organic components from water when a polar solvent is added to an aqueous salt solution. It preferentially solvates the water that was solvating ions, to give a "salting in" effect [64]. When a salt is added, it introduces ionic forces which alter the structure of the liquids in equilibrium. Molecules of water surrounding the ions become unavailable for the solution

of the non-electrolyte and it becomes “salted out” from the aqueous phase. It captures part of the water molecules that have been solvating the ions; hence, the salt crystallizes [65]. The common kosmotropic salts used for two-phase extraction technique of ionic liquids are includes cations such as (NH_4^+, K^+, and Na^+) and anions ($PO4^{3-}$, OH^-, and $SO4^{2-}$, CO_3^{2-}). The experimental data reveals that the salting out order of some salts are ranked as: $MgC1_2$ >NaCl> KC1 > LiCl> Na_2SO_4 > K_2SO_4 > $(NH_4)_2SO_4$ > $MgSO_4$> $NaClO_3$ > $NaNO_3$ [64-65].

SALLE is a sample pretreatment method as it is simplicity, rapid partition equilibrium, easy purification and condensation of analytes extracts [66]. When inorganic salts are added to a solution containing water and water miscible organic solvents two clearly separated phases are formed. Solvents such as acetone, acetonitrile, 1, 4-dioxane, tetrahydrofurane, l-propanol, and 2-propanol solvents show a clear phase separation [67- 68]. There are parameters that affect extraction efficiency including the kind and the amount of water-soluble organic solvents, the kind and the amount of inorganic salts, pH of the sample solutions are some among identified [68].

Although, several methods has been used for the quantitative determination of caffeine and nicotinic acid, simultaneous determination of caffeine and nicotinic acid using SALLE sample pretreatment was not yet reported. Therefore, the main objective of this study was to develop an analytical method of SALLE followed by HPLC for determination of the two compounds from coffee.

3. Materials and Methods

3.1 Chemicals

HPLC grade methanol (CH_3OH, 99.9%) and acetonitrile (CH_3N ,99.8%,mJehanger villa, India), acetone (C_3H_6O,Tianjin, china), 1-propanol (C_3H_8O, Sigima Aldrich, Germany), ammonium sulfate $(NH_4)_2SO_4$, phosphoric acid (H_3PO_4, 85%, Riedel-deHein, ACS, Germany), sodium chloride, (NaCl, 99.9%) sodium sulfate, (Na_2SO_4, 99.5%) sodium hydroxide, (NaOH, 99.3%) (Fisher scientific, UK), magnesium sulfate, ($MgSO_4$, Finken, USA), TBAHS, 97%, Sigima Aldrich, India) hydrochloric acid, HCl (Sigima Aldrich), caffeine, ($C_8H_{10}N_4O_2$, Sigima Aldrich, China) nicotinic acid ($C_6H_5NO_2$,99% , Navketan, India), and deionized water were used.

3.2 Instrument and Apparatus

3.2.1 Apparatus

Micropipettes (Hunda, China), hot plate magnetic stirrer (Bibby Sterilin, UK), pH-meter(HANNA, China), Oven (Widnes, M 50 C, England), Elma sonicator, Germany S10,Ge, Vortex (Fisher scientific, UK) centrifuge (1790xg,4000r/min), sieve 250mic (mesh, UK), Millipore deionizer (Rios D®3Uv, France), filter paper (whatman® no.542,110mm, England) electrical balance (Kern, ABJ-220NM, Germany), electric grinder (nima, Japan), were used.

3.2.2 Instruments

PerkinElmer HPLC quaternary solvent systems (Shelton, USA) equipped with Flexar solvent manager, Flexar LC auto sampler, Flexar LC pump, Flexar column oven, and Flexar UV/VIS detector, was employed to perform chromatographic analysis. A C18 analytical column (150 x 4.6 i.d., particle size 5 µm) for separation of analytes. Data acquisition and processing were achieved using chromera software (4.1.16396) throughout the analysis.

3.3 Sampling and Sample Collection

The coffee sample was collected from southwest regions of Oromia (Ilu Abba Bora and Jimma).

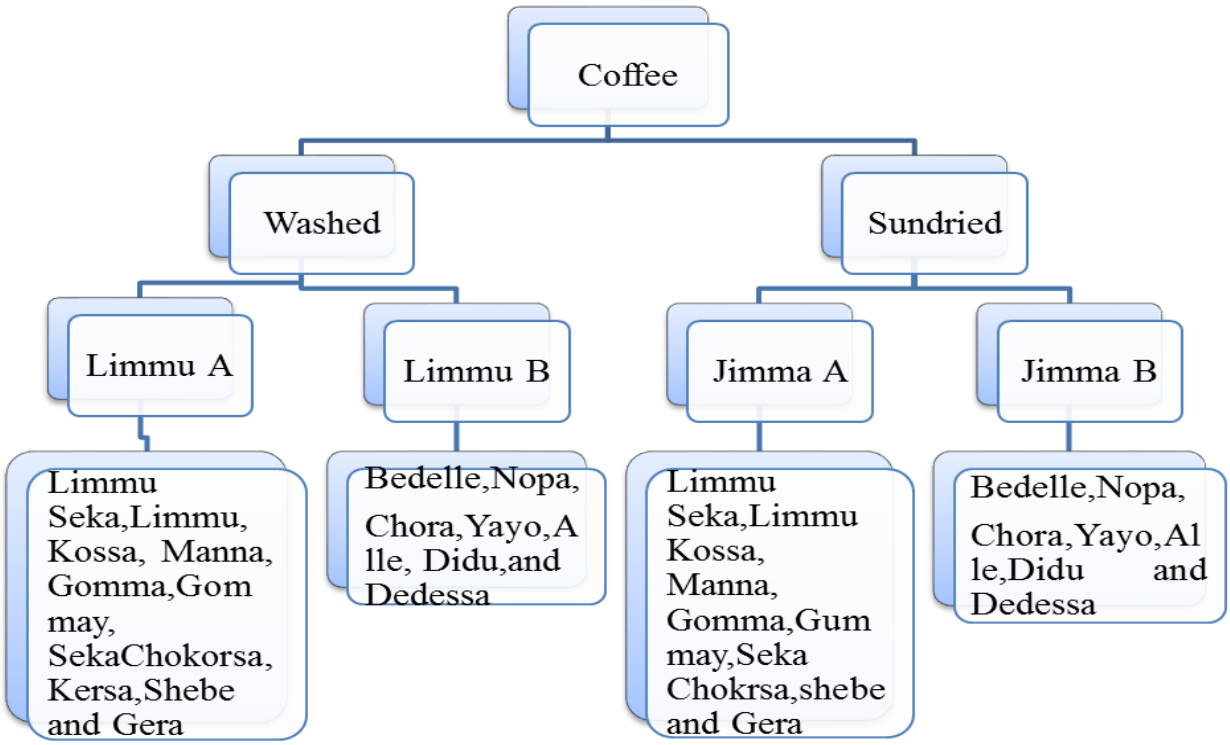

Figure 3.1 Schematic category of coffee in Southwest regions of Ethiopia [69]

Based on the category made by ECX, the sample was taken by lottery method from the two Oromia zones Jimma and Ilu Abba Bora. The specific location of the two woredas in the Zones were shown in the figure 3.2.

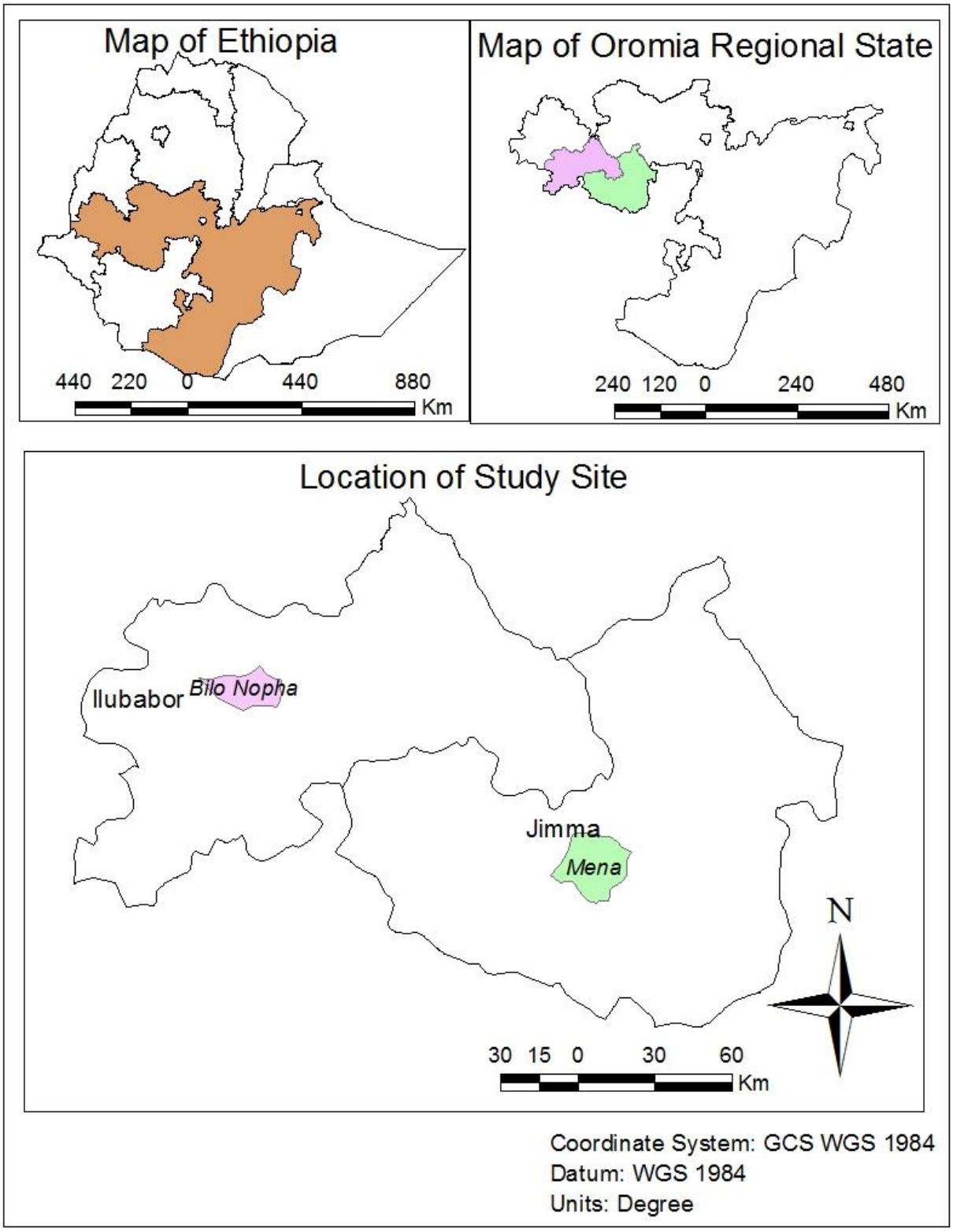

Figure 3.2 Location of study site

Among the coffee groups, sundried each 1 kg of Jimma A (Manna) and Jimma B (Nopa) were taken randomly. The sample was transported with poly ethylene bag and kept in the laboratory till analysis. The sample was washed carefully with deionized water and divided into two, in to raw and roasted. The raw sample was dried with low temperature in an oven to assist grinding and study at dry bases. Both raw and roasted coffees were ground with electric grinder and sieved to get uniform sized particles.

3.4 Standard solution preparations

The standard stock solution of 2000 ppm caffeine and nicotinic acid were prepared by weighting 0.02 g of each in 100 mL volumetric flask using deionized water. Then, a mixture of an intermediate solution was prepared in 100 mL volumetric flask with concentration of 100 and 1000 ppm of nicotinic acid and caffeine respectively.

3.5 Chromatographic Conditions

An isocratic elution of binary solvent system was employed with 30% methanol (B) and 70% buffer (D) prepared of 0.02 M H_3PO_4 adjusted to a pH four with 0.1 M NaOH and 1 M HCl using RP-HPLC C_{18} column. Analysis was performed with a flow rate of 1.2 $mLmin^{-1}$, using a column temperature of 25 ℃ and an injection volume of 10 µL monitoring wave length at 260 nm with total run time set at 5 min. Peaks identification was done by injection of individual standards and their elution time was recorded.

3.6 Procedure for Optimization of Parameters

From an intermediate solution prepared it was further diluted to a low concentration for optimization of type of organic solvent, amount of organic solvent, type of salt , amount of salt, pH, and ion pair concentration. The extraction was made by adjusting the pH of buffer at four, a salt and an ion paring was added. Then shaked gently, vortexed for 30s, and centrifuged for 5 min. A 1 mL supernatant is transferred in to a 10 mL beaker and dried in an oven at a temperature of 80-90 ^{0}C. Finally reconstituted with 2 mL deionized water and subjected to HPLC for analysis.

3.6.1 Procedure for Extraction of Real Sample (SALLE procedure)

The ground sieved coffee, 0.5 g of each raw and roasted were extracted with hot boiled distilled water at 80-90 ^{0}C on water bath in beaker with 25 mL of distilled water for 5 min. Then the extract was cooled to room temperature and filtered with Whatman No. 42 filter paper. A 1 mL of the filtrate was transferred into 10 mL bottom conical shape plastic test tube and diluted filling with deionized water to the mark. 5 mM of TBAHS (tetra butyl ammonium hydrogen sulfate) was added shaking gently and kept for 2-3 min. 2 mL of acetonitrile was added for extraction followed by 5 g of $(NH_4)_2SO_4$ for salting out. The mixture is whirled till the salt is completely dissolved, vortexed for 30 s to ensure mixing, and centrifuged at 4000 rpm (7350×g) for 5 min. to get a clear and good phase separation. Then, 1 mL of supernatant was transferred into 10 mL beaker and dried in an oven at 85-90^{0}C. After dried, reconstituted with 2 mL of deionized water, filtered with 0.22 µm teflon syringe filter into HPLC vials for injection.

4. Results and Discussion

In this study HPLC conditions and the effect of different important parameters influencing the extraction efficiencies including the water-miscible organic solvents (type and amount), salt (type and amount), pH of aqueous sample, and amount of ion pair was investigated.

4.1 Optimization of HPLC Conditions

Different binary mobile phases was tested, including acetonitrile/water, methanol /water, acetonitrile/ buffer, and methanol /buffer at different percentages of compositions. As conciliation for suitable retention times for the target analytes and a better sensitivity, methanol (solvent B) and buffer (solvent D) containing 5 mM of TBAHS were chosen as the mobile phase. The effect of injection volume was investigated at 10, 15, 20 and 25 µL. It was observed that the peak area increased as a volume increased. However, the peak area becomes broad as injection volume increases. Hence, 10 µL was chosen comparatively based on sharpness and sensitivity of peaks. The effect of mobile phase flow rate was evaluated at 1, 1.2, 1.4 and 1.5 $mLmin^{-1}$. Retention time and peak width was reduced as flow rate increases. As verification 1.2 $mLmin^{-1}$ was chosen due to better sharpness and resolution than the others. The column temperature was set at 25 ℃ and wave length 260 nm during the entire work.

4.2 Optimization of the SALLE Procedure

In a SALLE procedure, several factors affect extraction efficiency such as the type and volume of the organic solvent, type and amount of salt, pH of the sample and effects of TBAHS concentration and volume, were studied [70].

4.2.1 Type of Extraction Solvent

When salt is added to water miscible organic solvents there is a competition between the non-electrolyte and electrolyte solute for aqueous phase, causing non-electrolyte (organic solvents) to move away from the aqueous phase and separation of the organic phase and aqueous phase was occurred [71]. To get an optimum solvent, the efficiency of organic solvents such as; acetonitrile, methanol, propanol, ethyl acetate, and acetone were examined. Of all these better extraction efficiency with clear phase separation was observed when acetonitrile was used.

It is may be due to high polarity of acetonitrile and similar index value of acetonitrile and water (10.2 and 5.8) respectively [71]. Thus, acetonitrile was selected throughout the entire work.

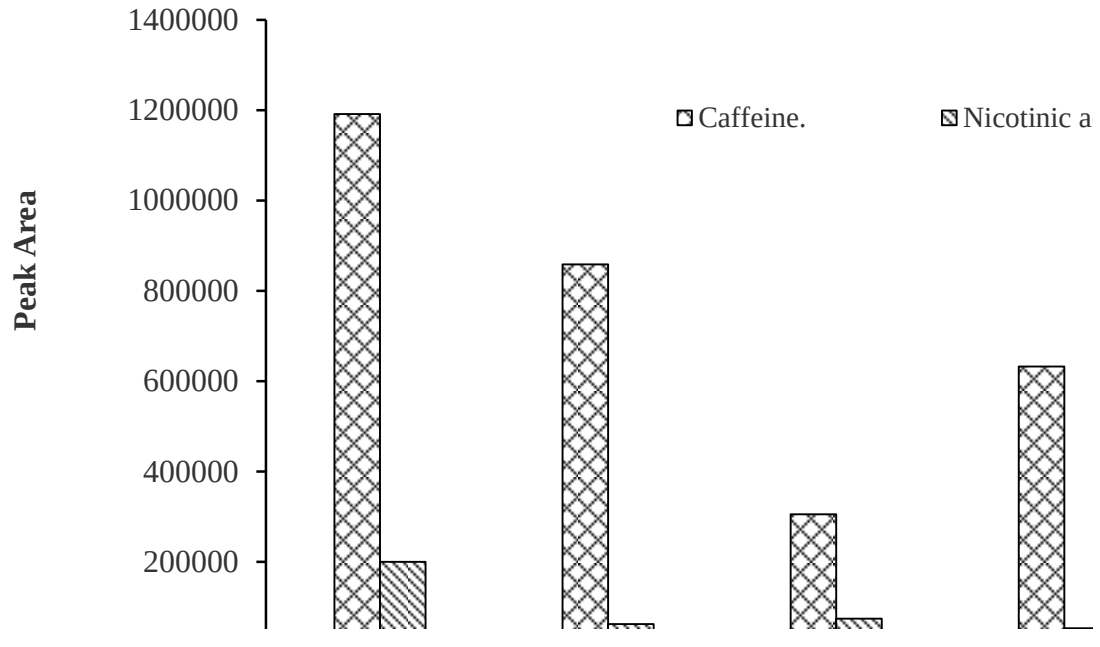

Figure 4.1 Type of extraction solvent. Experimental conditions: concentration of TBAHS, 5mM; volume of TBAHS, 2 mL; sample pH,4; salt concentration, 50% $(NH_4)_2SO_4$; vortex time, 30 s; centrifugation rate and time, 4000 rpm and 5 min respectively.

4.2.2 Effect of Extraction solvent Volume

The volume of organic solvent is one of the important parameter that influence the extraction performance of SALLE [72]. To attain an optimal volume, different volumes of acetonitrile ranging from 1- 3 mL were tested. At low volume, phase separation is not clear and sufficient to take. But at higher volume a clear and sufficient phase separation takes place making convenient to collect the organic layer. However, around 2 mL is an optimum volume to collect and get a clear phase separation. Since only 1 mL of the extract was taken and dried in an oven as the volume increased it takes long time to dry.

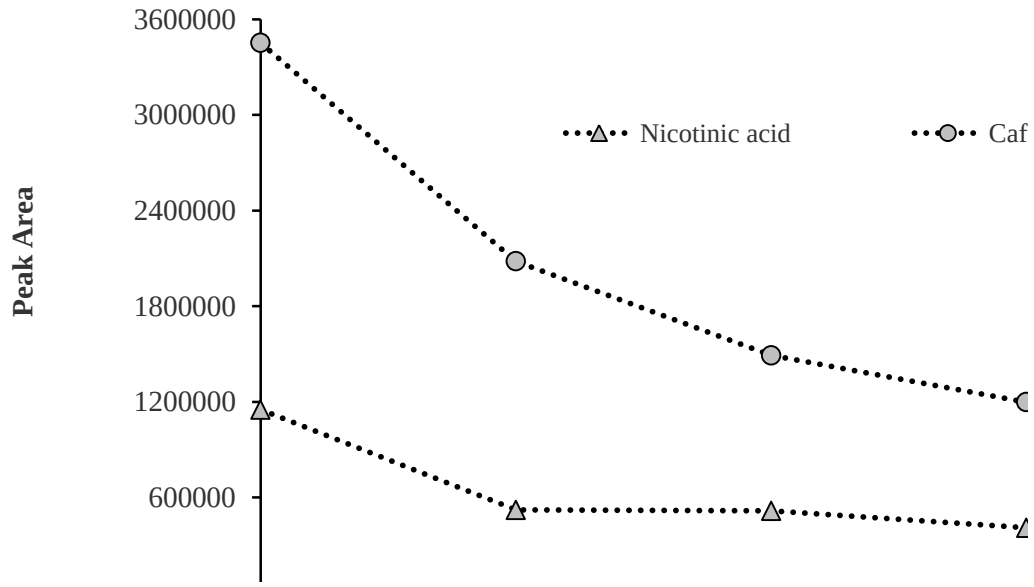

Figure 4.2 Effect of extraction solvent. Experimental conditions: concentration of TBAHS, 5 mM; volume of TBAHS, 2 mL; sample pH,4; salt concentration, 50% $(NH_4)_2SO_4$; vortex time, 30 s; centrifugation rate and time, 4000 rpm and 5 min respectively.

4.2.3 Effects of Type of Salt

In salting out, different salts have different degrees of phase separation when added to water miscible organic solvents [73]. Hence, the effects of some salts ($(NH4)_2SO_4$, $MgSO_4$, Na_2SO_4 and NaCl) were examined in this study. Magnesium sulfate shows good efficiency for only nicotinic acid but, poor for caffeine and also solubility and amount of supernatant volume is low which makes it difficult to take an optimum. Although all salts causes phase separation $(NH_4)_2SO_4$ was selected due to a better solubility, phase separation a good reproducibility and was used as a salting-out reagent throughout this work (figure 4.3).

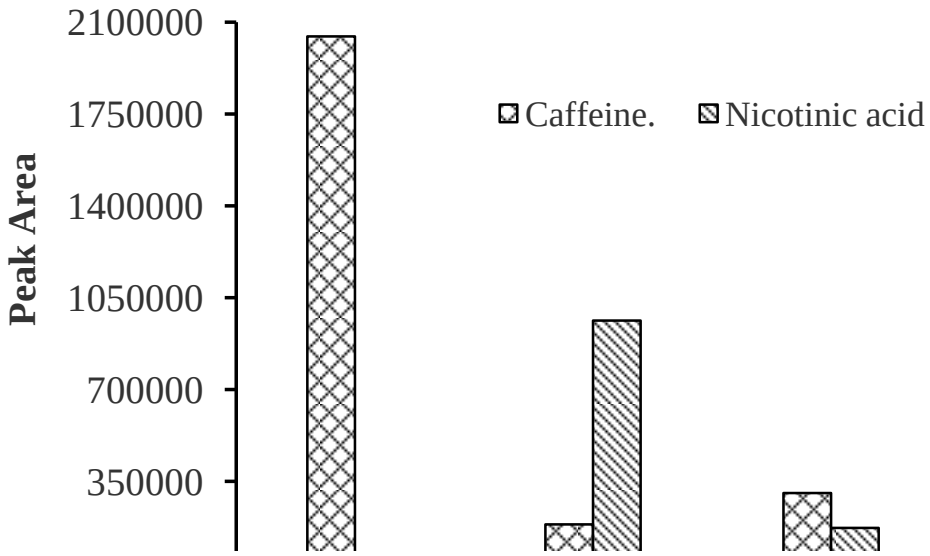

Figure 4.3 Effect of Type of salt. Experimental conditions: concentration of TBAHS, 5 mM; volume of TBAHS, 2 mL; sample pH,4; salt concentration, 50% $(NH_4)_2SO_4$; vortex time, 30 s; centrifugation rate and time, 4000 rpm and 5 min respectively.

4.2.4 Effects of Amount of Salt

In SALLE, concentration of the salt has induced a great influence on phase separation. Largely, high concentration of salt would enhance the phase separation and increase the extraction performance of analytes [74]. The effects of the amounts of salting out reagent $(NH_4)_2SO_4$ was evaluated by adding different a mounts in the range 2-6 g (20-60%) into the aqueous sample solutions containing analytes. When the amounts of $(NH_4)_2SO_4$ increased from 2-6 g, the extraction recoveries had slightly increases. However, on further increasing of the amounts of $(NH_4)_2SO_4$ nearly had no influence on the extraction efficiency, but as the amount of salt increases the solubility in 10 mL is not entirely completed. So 5 g (50%) was selected as the amounts of salting out reagent $(NH_4)_2SO_4$ for this work (figure 4.4).

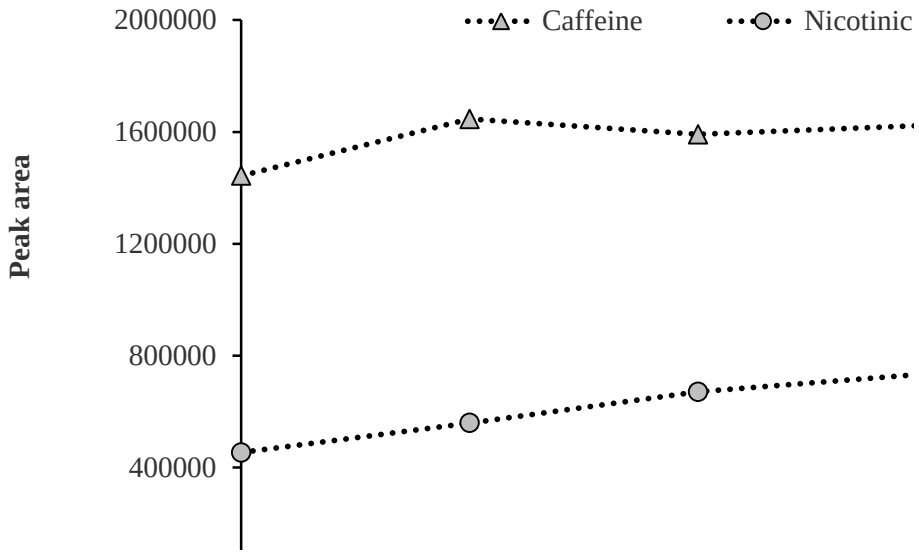

Figure 4.4 Effect of Amount of salt. Experimental conditions: concentration of TBAHS, 5mM; volume of TBAHS, 2 mL; sample pH,4; salt concentration, 50% $(NH_4)_2SO_4$; vortex time, 30 s; centrifugation rate and time, 4000 rpm and 5 min. respectively.

4.2.5 Effect of the pH of Sample

In SALLE, chromatographic separation of charged analytes had been achieved by ion suppression (the careful adjustment of the mobile phase pH to result in a non-ionized analyte) [75]. It plays a vital role in affecting the extent of ionization as well as the solubility of ionizable organic compounds. In case of ionizable organic compounds the sample solution should be rather acidic in order to facilitate the extraction of the neutral molecular forms with the organic solvent [76]. The effect of sample pH was examined varying its value from 2 - 8 using 0.02 M H_3PO_4 buffer adjusted using HCl and NaOH. The peak areas of the target analyte was increased with the rise in pH of the sample solution. A complete transfer of the analytes to the acetonitrile phase occur when the analyte is neutral. The experimental data revealed that high peak area was obtained at pH 4 and 6, but a good peak nature is seen at pH 4 and this was selected as the optimum for the further work (figure 4.5).

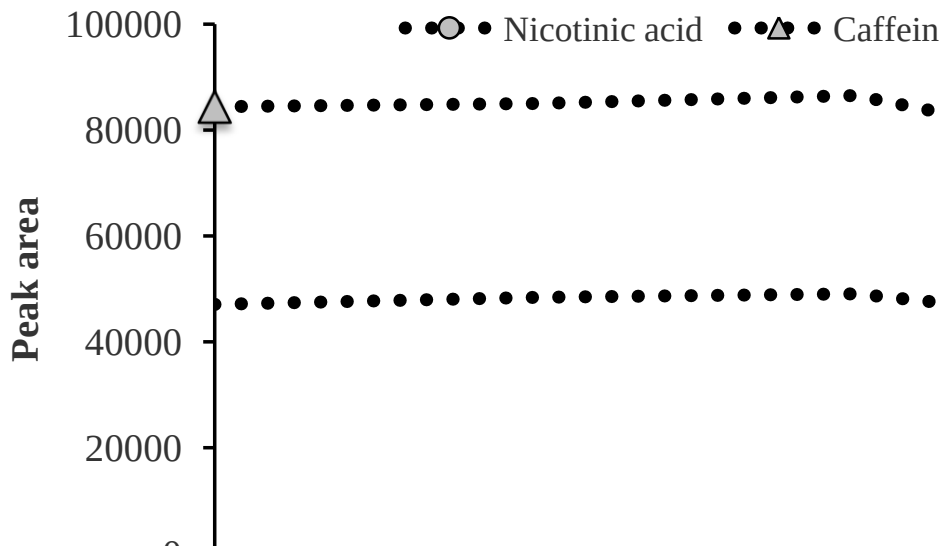

Figure 4.5. Effect of the pH of sample. Experimental conditions: concentration of TBAHS, 5 mM; volume of TBAHS; acetonitrile volume 2 mL; salt concentration, 50% $(NH_4)_2SO_4$; vortex time, 30 s; centrifugation rate and time, 4000 rpm and 5 min respectively.

4.2.6 Effects of TBAHS Concentration and Volume

Many ionic compounds are weakly retained, if at all, in reversed-phase HPLC with common aqueous organic mobile phases without ionic additives [77]. Ionic species, ion-pairing reagents, inorganic salts and other competing ions can influence the signal of the target compound. Ion paring technique improves separation providing reduced retention times, highly results in sharper peak shapes in the separation of charged analytes [75, 77].

The effect of concentration of the TBAHS on the extraction efficiency of ion paring LLE was evaluated over the range of 0-20 mM, at a concentration of 1 M. It was observed that the extraction efficiency of the target analyte was found to increase in the volume range of 0-15 mM and then start to decline. The reason for the decrease of the extraction efficiency at higher volumes of ion-paring reagent used was the fact that the target analytes are unable to form ion paring as a result of steric hindrance from the side chain of its structure. Hence, 5 mM was selected as optimum volume for the entire work (figure 4.6).

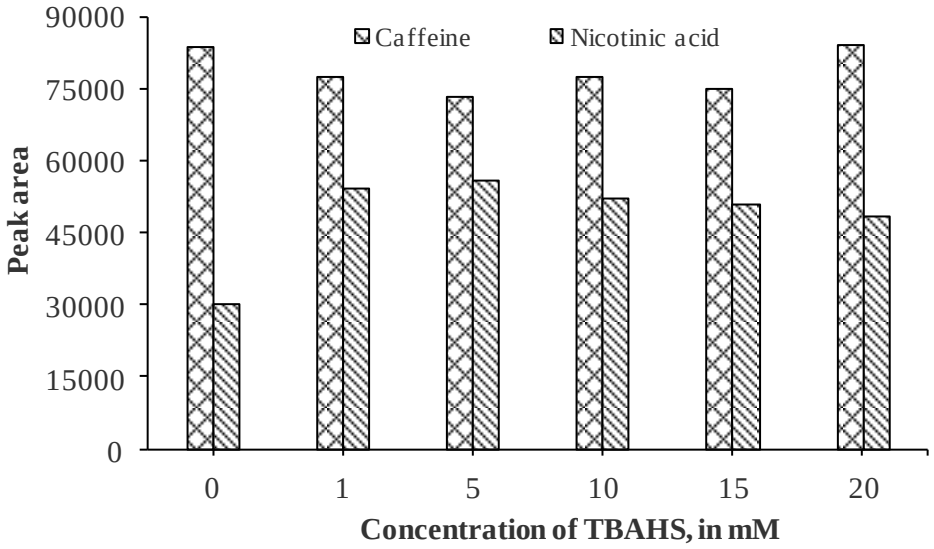

Figure 4.6. Effect of IP concentration. Experimental conditions: volume of TBAHS, 5 mM ; volume of acetonitrile, 2 mL; sample pH, 4; salt concentration, 50% $(NH_4)_2SO_4$; vortex time, 30 s; centrifugation rate and time, 4000 rpm and 5 min. respectively

4.3 Validation of the Proposed Methods

4.3.1 Calibration Curves and Analytical Performance Characteristics

The developed method of SALLE followed by RP-HPLC-UV detector was evaluated using calibration curve which was established using raw and roasted coffee samples. The calibration curves were constructed by spiking coffee with a mixture standard caffeine and nicotinic acid at seven concentration levels: (1, 2.5, 5, 10, 25, 50, and 100 mgL^{-1}) and (0.1, .25, 0.5, 1, 2.5, 5, and 10 mgL^{-1}) in which each level was extracted in duplicate approach at the optimum conditions. Each duplicate extract was then injected in duplicate manner. Calibration curves were constructed using the peak areas as instrumental responses versus concentration of analytes. The coefficient of determination (R^2) obtained for nicotinic acid and caffeine were 0.9953 and 0.9999 respectively showing acceptable linearity over the selected ranges.

4.3.2 Limit of Detection and Limit of Quantification

The LOQ is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy and LOD is the lowest concentration of analyte that can be detected,

but not necessarily quantified [78]. It can be considered as the minimum analyte concentrations by a rule of signal-to-noise ratio (LOD=3S/N, LOQ=10S/N). [79]. Based on signal-to-noise ratio the LOD of the proposed method is 0.2 and 0.8 $mgkg^{-1}$, LOQ is 0.5 and 2.5 $mgkg^{-1}$ for nicotinic acid and caffeine respectively. The statistics of importance of the developed method is summarized in Table 4.1.

Table 4.1 Table of linearity range, LOD, LOQ, and calibration equation.

Analyte	Calibration equation	Linearity range(mgL^{-1})	R^2	LOD ($mgkg^{-1}$)	LOQ ($mgkg^{-1}$)
Nicotinic acid	y = 12471x + 10181	0.5 - 5.0	0.9953	0.2	0.5
Caffeine	y = 41711x + 8174	2.5 – 100	0.9999	0.8	2.5

4.3.3 Precision Study

The precision of the developed method was studied in terms of repeatability (intra-day precision) and reproducibility (inter-day precision) using the optimized conditions to raw and roasted coffee. Repeatability is obtained when all measurements are made by the same analyst during a single period of laboratory work, using the same solutions and equipment's and reproducibility is obtained under any other set of conditions, including that of between analyst or laboratory session for the same analyst [80].

Table 4.2 Intra- and inter-day precisions of the proposed method (% RSD) for spiked coffee samples

Analyte	Intra-day (n = 4)		Inter-day (n = 10)	
	Level 1	Level 2	Level 1	Level 2
Nicotinic acid	6.1	5.1	9.7	1.5
Caffeine	4.4	1.1	2.3	5.7

Level 1: *10 µL for nicotinic acid and caffeine (0.1 mgL^{-1} for nicotinic acid and 1 mgL^{-1} for caffeine)*
Level 2: *50 µL for nicotinic acid and caffeine (0.5 mgL^{-1} for nicotinic acid and 5 mgL^{-1} for caffeine)*

Repeatability of the method was evaluated by extracting spiked deionized water at two concentration levels where each was prepared in duplicate and then injected in duplicate on the same day, under the same experimental conditions. Likewise, reproducibility was investigated with spiked deionized water extracted at two concentration levels each prepared in duplicate and injected in duplicate for

five consecutive days. Intra- and inter-day precision results obtained were expressed using the relative standard deviations (% RSD) of the peak areas.

4.3.4 Applications of the Method and Recovery Studies

The applicability of the method was investigated by performing recovery studies in raw and roasted coffee samples in order to check the trueness in these matrixes. The sample was spiked at two concentration levels, extracted and injected in duplicate approach as previously used for the precision study. Then relative recoveries was calculated comparing the average peak area for the analytes in water samples with peak of corresponding spiked samples of raw and roasted coffee. When as blank sample (deionized water) was analyzed with developed method but, none of the two target analytes were detected. However, relative recoveries and the corresponding % RSD of each target analytes of samples are shown in Table 4.3. The relative recoveries obtained with the proposed method was in the range of 82-122%, in the raw and roasted coffee sample. Thus, the results obtained with the proposed method was within the acceptable range.

Table 4.3 Average recoveries of roasted and raw coffee with (%RSD)

	Coffee type			
Analyte	**Manna Coffee**		**Nopa Coffee**	
	Roasted	**Raw**	**Roasted**	**Raw**
Nicotinic acid	82 ± 13.2	87 ± 18.3	109 ± 13.6	102 ± 6.1
	99 ± 9.7	97 ± 9.7	86 ± 9.7	97 ± 0.6
Caffeine	84 ± 0.7	99 ± 0.4	105 ± 0.7	84 ± 4.4
	82 ± 1.6	122 ± 1.5	94 ± 1.6	83 ± 1.1

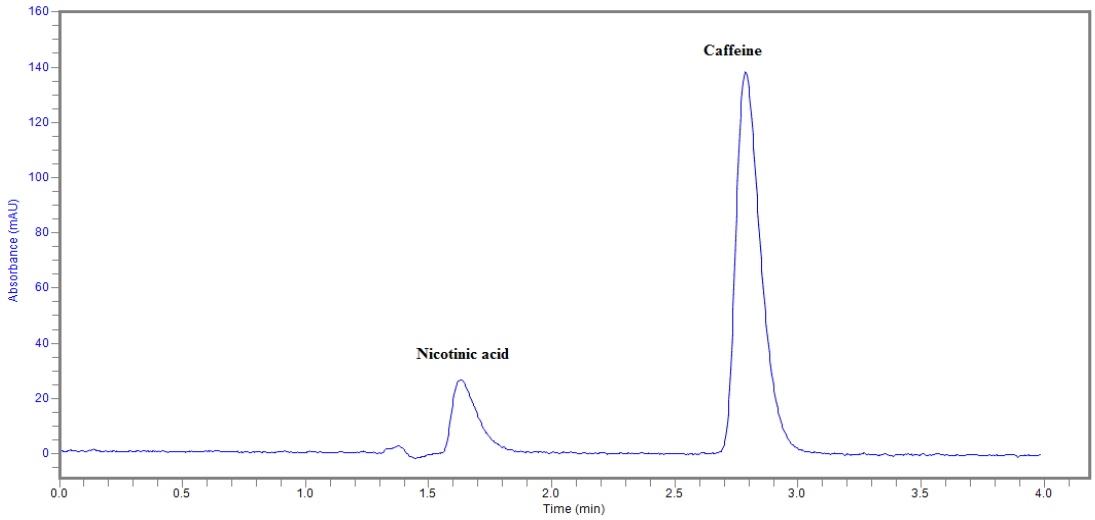

Figure 4.7 Chromatogram of the standard nicotinic acid and caffeine in water under chromatographic conditions: methanol 30% and buffer 70% containing 5 mM of TBAHS, injection volume 10 µL, column temperature 25 ^{0}C, and wave length 260 nm.

The retention time of nicotinic acid 1.6 and caffeine 2.9 was recorded respectively when run under these optimized conditions. The chromatogram of nicotinic acid and caffeine appeared in the same retention time of the standard when applied to roasted coffee under the same chromatographic conditions.

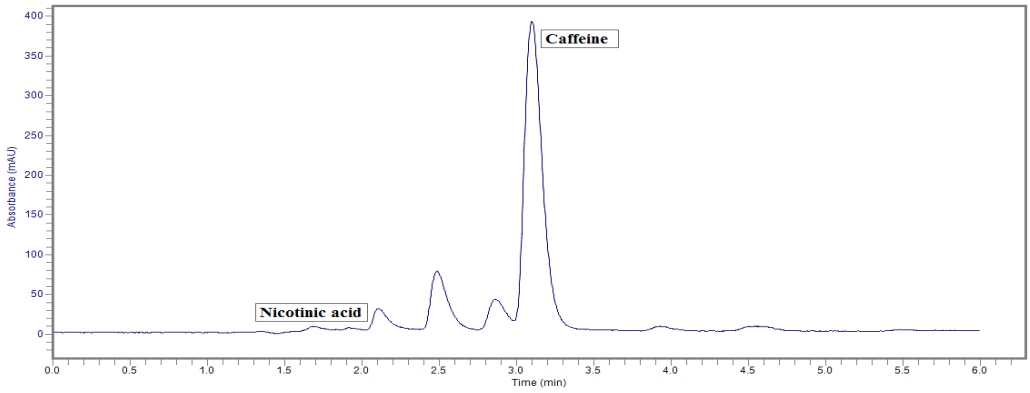

Figure 4.8 Chromatogram of roasted coffee under chromatographic conditions, refer to figure 4.7

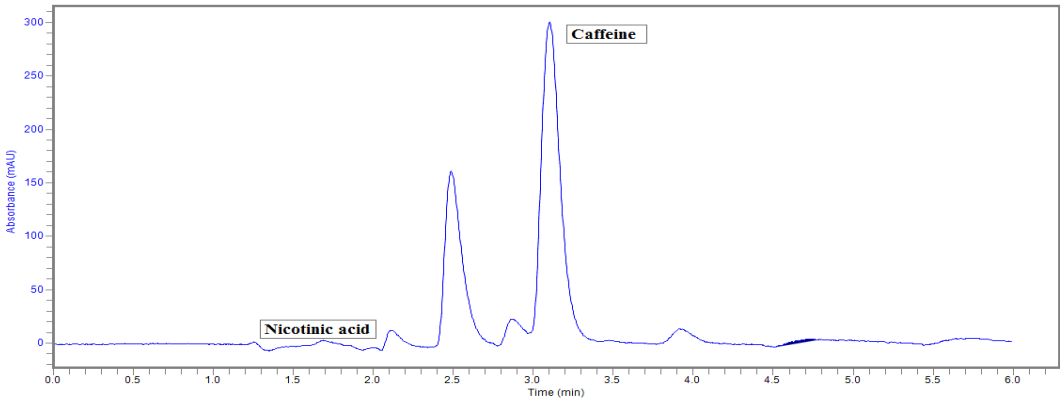

Figure 4.9 Chromatogram of raw coffee under chromatographic conditions refer to figure 4.7

Similarly, the chromatogram of nicotinic acid and caffeine appeared in the same retention time of the standard when applied to a raw coffee under the same chromatographic conditions.

4.3.5 Comparison with other Methods

The developed SALLE procedure followed by HPLC has been compared with other recently reported methods for simultaneous determination of caffeine and nicotinic acid. In comparison of the proposed method with different techniques such as HPLC-VWD, LC–MS, and RP-HPLC-UV were seen including some parameters linearity range, LOD, LOQ, RR, R^2 and % RSD. Details of the comparison are shown in Table 4.4. From the observed comparability, the developed method has a good linearity, relative recovery, a wider range and also other parameters are in an acceptable range showing performance and applicability of the method to be utilized for determination of caffeine and nicotinic acid.

Table 4.4 Comparison of the proposed method with other reported methods for the extraction and determination of nicotinic acid and caffeine

No	Method	Sample preparation	Analytes	Linear range (mgL^{-1})	LOD ($mgkg^{-1}$)	LOQ ($mgkg^{-1}$)	%RSD	%RR	R^2	Ref.
1	HPLC-VWD	EC machine	NA and Caffeine	10-250	0.03–0.06	0.1–0.2	< 5	71–92	0.9985	[81]
2	LC–MS	SE	NA and caffeine	0.1-10	11.9	-	< 5	89.6- 105.1	0.999	[53]
3	RP-HPLC-UV	SE	NA and caffeine	-	0.01	-	-	89- 104	0.995	[14]
4	HPLC-MAE	MAE	NA and caffeine	-	0.02	0.05	-	-	1	[82]
5	HPLC-DAD	SE	NA and caffeine	0.01-500	-	-		84-99	-	[83]
6	RP-HPLC-UV	SALLE	NA and caffeine	0.5-5.0 2.5-100	0.02 0.8	0.4 1.6	<10	82-122	0.9953 0.9999	This work

Note: NA: Nicotinic Acid; SE: Solvent Extraction; MAE: Microwave assisted Extraction; EC: Espresso *coffee*

5. Conclusion and Recommendation

5.1 Conclusion

The way coffee is processed and its natural compositions are significant factors for measure of coffee quality. Caffeine and nicotinic acids are among coffee composition contributing to coffee quality. In the present study, a new analytical method has been proposed for sample preparation and quantitative determination of caffeine and nicotinic acid using SALLE in combination with RP- HPLC-UV. Different parameters affecting the chromatographic separation including (mobile phase composition, flow rate, wave length, injection volume, and column temperature) as well as the extraction efficiencies of the target analytes (extraction solvent type and volume, salt type and amount, pH, concentration of ion pair) were examined and the optimum conditions were identified. The proposed method, under optimized conditions gives a good determination of the target analytes with a linearity range of 0.5-5.0 and 2.5-100 mgL^{-1}, LOD, 0.2 and 0.8 $mgkg^{-1}$, LOQ, 0.5 and 2.5 $mgkg^{-1}$, R^2, 0.9953 and 0.9999 respectively for nicotinic acid and caffeine. The method is within acceptable precision (% RSD < 10) and a relative recoveries in the range of 82-122%. Hence, the developed method result revealed that it could be alternatively used as it is a simple, uses less toxic and environmentally friendly solvents, fast extraction and analysis time for simultaneous determination of caffeine and nicotinic acid from coffee and related matrixes.

5.2 Recommendations

Based on the findings of this study, the chromatographic conditions and some of the analytical performance of the developed method for the determination of caffeine and nicotinic acid was successfully addressed. However, the roasting temperature effect on the concentration of caffeine and nicotinic acid in the coffee are not addressed in this study due to lack of temperature controller roasting. Since water soluble composition of coffee (chlorogenic acid, trigonelline and sucrose) and water insoluble (kahweol and casfestol) are also important components of coffee that affect cup quality, further work has to be done to develop and validate HPLC methods to determine these components, either individually or simultaneously for coffee quality. So, to determine the level caffeine and nicotinic acid other researchers can apply the developed method for further investigation.

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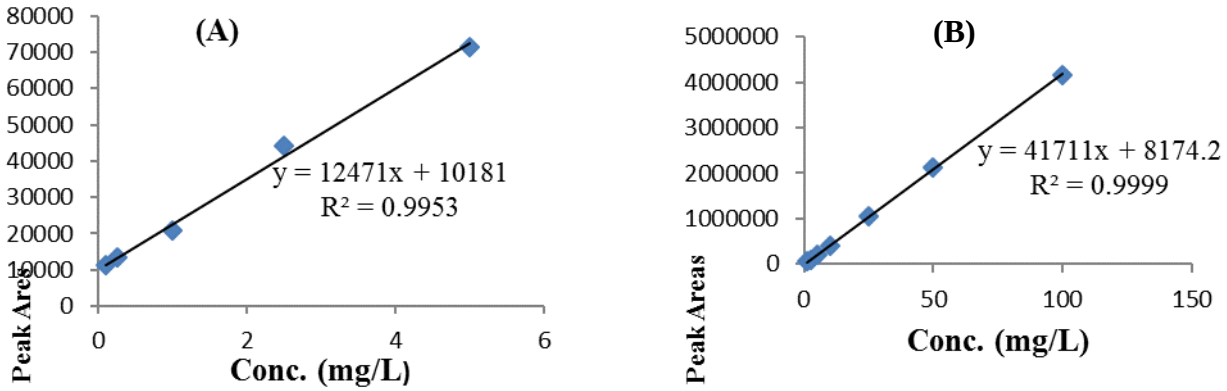
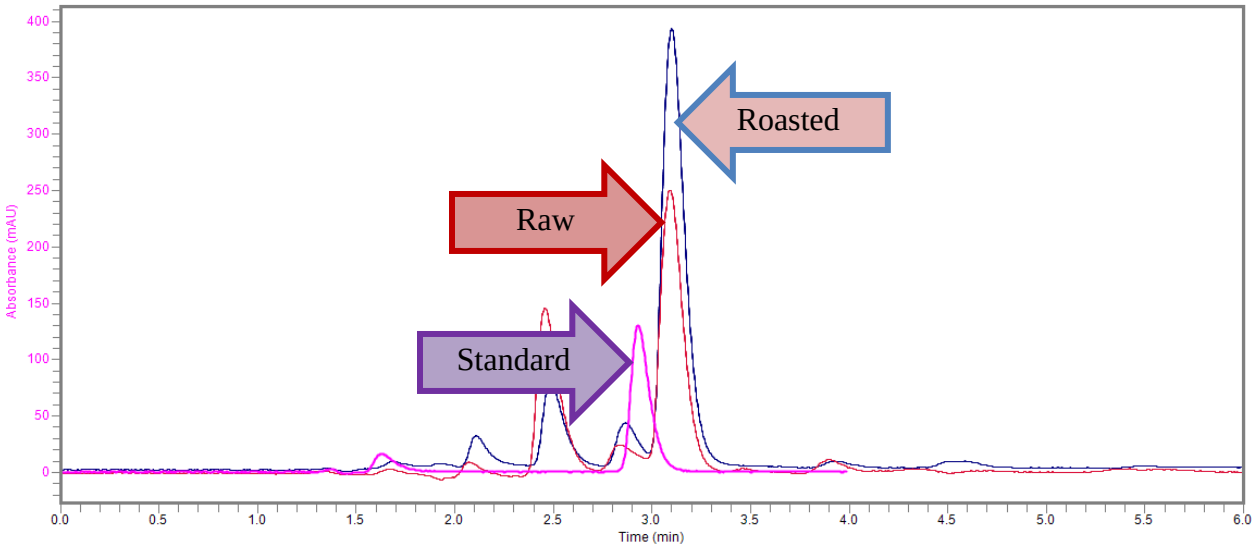
Appendixes

Appendix I. Calibration curve of caffeine and Nicotinic acid



Calibration graph of nicotinic acid (A) and calibration graph of caffeine (B)

Appendix II. Overlay graph of Standard, raw, and roasted coffee



Appendix III. Photos of some coffee types and laboratory activities

Raw coffee

Roasted coffee

Ground raw coffee

Ground raw roasted coffee

Buffer adjustment

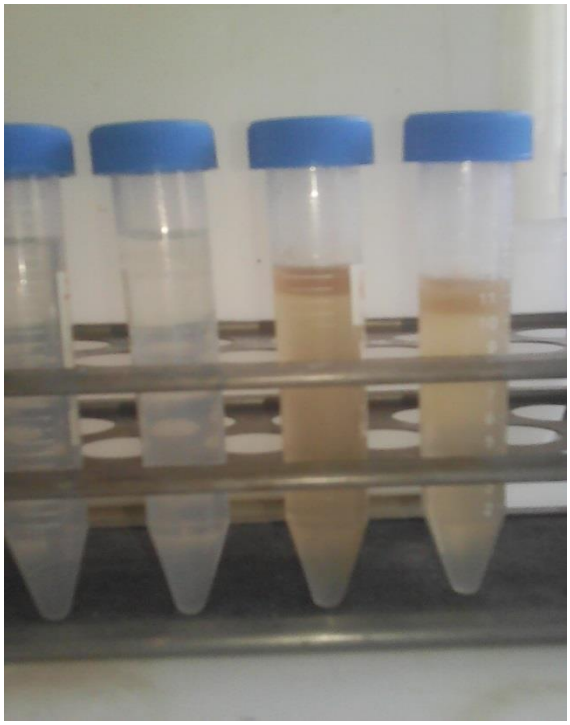
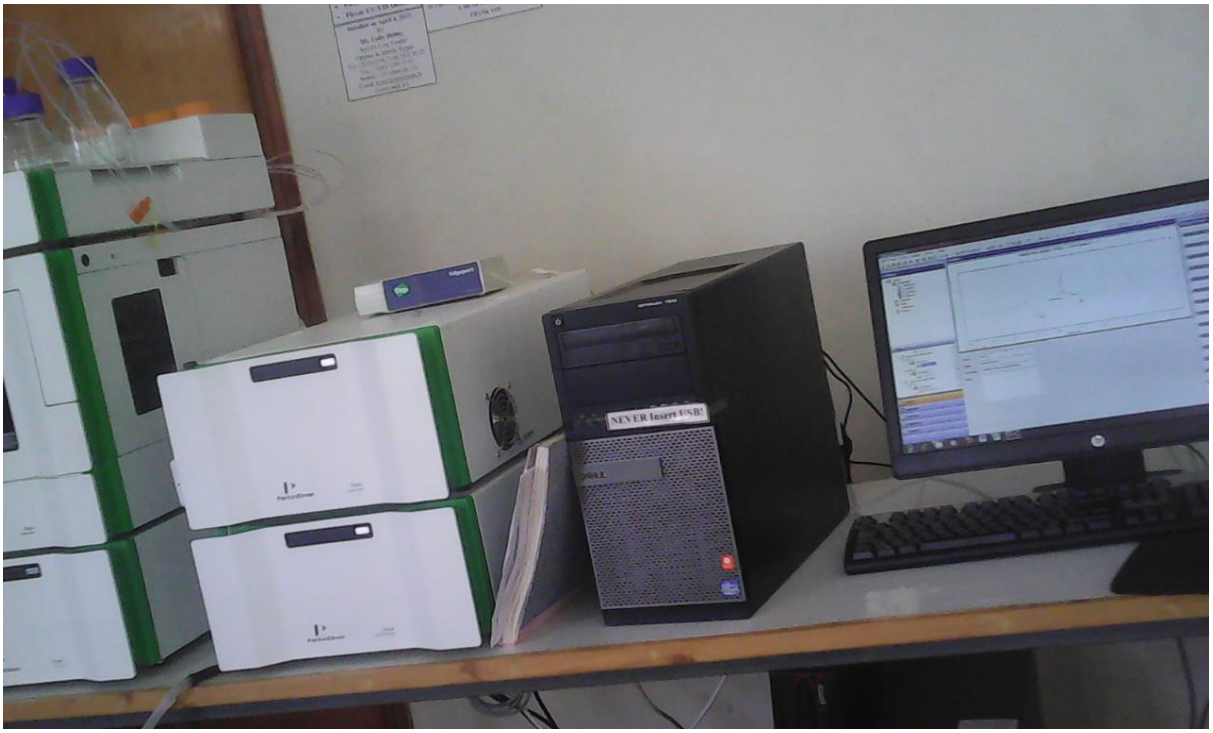


Photo of phase separated sample



PerkinElmer HPLC quaternary solvent system