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SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF CHEMISTRY**



Determination of Caffeine Level in Coffee

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Determination of Caffeine Level in Coffee

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Abstract

Coffee is an important commodity culturally, commercially and economically in the world. Because of its consumption in most countries in the world, it is important to investigate the exact amounts of its chemical constituents. Caffeine is one of the main components of coffee that affect the quality of coffee, both before and after processing. In this project, rapid, simple and sensitive quantitative thin-layer chromatography and proton nuclear magnetic resonance spectroscopy methods for the quantification of caffeine in coffee, tea and soft drinks were developed and validated. The methods involved extracting samples with chloroform by sonication, and analysis by Camag TLC scanner 3 and quantitative proton NMR (qHNMR).

In the first method, Camag TLC scanner 3 was used for densitometric scanning and analysis in absorbance mode at 276 nm. The system was found to give a sharp peak for caffeine at R_f value of 0.64. The linear regression analysis data for calibration plots showed good linear relationship with $r^2 = 0.999$ in the concentration range of 200-1600 ng spot⁻¹ with respect to peak area. The method was validated for accuracy, precision and recovery.

In the qHNMR analysis, the method was based on 400 MHz proton NMR spectra of caffeine and theophylline (internal standard). Quantitative analyses were based on the integral ratio of selected signal belonging to the analyte with respect to that of an internal standard. The linear regression analysis data for calibration plots showed good relationship with $r^2 = 0.999$ in the concentration range of 0.4-2.0 mg/mL with respect to peak area (integration). The recovery studies performed on pre-analyzed samples ranged between 97-108%.

The caffeine values in coffee, tea and soft drink samples, quantified by both methods, were found to be in agreement with those reported in the literature. Caffeine contents were found to be: in green and roasted coffees (0.8 - 1.1%), tea (1.9 - 2.1%), soft drinks (5 - 7 mg per 100 mL), machine-brewed coffees (80 - 103 mg per 150 mL) and home-brewed coffees (Ethiopian traditional style) 12 – 39 mg per 150 mL. Both methods can be recommended for routine analysis.

1. Introduction

1.1. Importance of Coffee in World trade

Coffee is the most important traded commodity in the world after oil [10]. It is also one of the most popular beverages in the world for its characteristic taste, aroma and for its potential beneficial effects on human health [2, 9]. The name coffee probably originated from the name of the province 'Keffa' where shepherds from Abyssinia/Ethiopia discovered the coffee beans in the 6th century [14]. In the 13th century coffee's restorative powers were known and spread throughout the Islamic world. Two hundred years latter coffee was sold in Europe, thus introducing the new beverage into Western life and custom [14].

The popularity of coffee as a beverage is increasing, despite the fact that there are reports that it is not necessarily good for human health [9]. There have been numerous reports on diseases associated with coffee consumption [10, 14]. Recent knowledge has put coffee in more positive light, and more and more reports suggest that coffee consumption has beneficial effects on human health [9, 10, 13, 15].

Coffee is commercially available as one of two varieties known as *arabica* and *robusta*. The coffee beverages are made from *arabica* and *robusta* roasted beans or blends of these two. Arabica coffees are generally considered of better quality and consequently they command higher prices. By taking this into account and considering that in recent times there is an increasing practice of selling coffees based on their variety and/or geographic origin, it is important to have methods to characterize these two varieties.

1.2. Chemical Constituents of Coffee

Coffee consists of the dried ripe seeds of *Coffea arabica*, *C. canephora*, *C. liberica*, or other *Coffea* species (*Rubiaceae*). Systematics experts have described over 80 species [44]. The plants are evergreen trees, widely cultivated in various parts of the world.

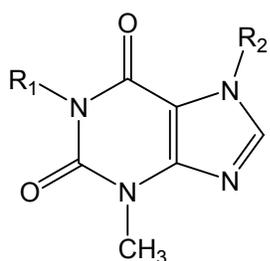
During processing, the fruit is deprived of its coat, then dried and roasted to develop its characteristic color, odor and taste. Coffee seeds contain 1-2% of caffeine (1) and traces of theobromine (2) and theophylline (3). These are mainly combined in the green seed with 5-7% chlorogenic acid (4), quinic acid (5), caffeic acid (6), trigonelline (7) and sucrose (8) [37]. Caffeine, chlorogenic acid, trigonelline and sucrose are the typical compounds in coffee that are relevant for flavor and/or bioactivity [1, 2, 7].

Caffeine (1) is a natural alkaloid found in coffee beans, tea leaves, cocoa beans and other plants [14, 18]. It is a xanthine derivative known to stimulate the central nervous system and is generally associated with improvements in alertness, learning capacity and exercise performance when moderately consumed [2, 3, 5]. Caffeine is one of the best central nervous system stimulants, and has weak diuretic action. It also has a characteristic bitter taste which is responsible for flavor formation [3, 8]. Other common members of this class with various physiological effects include theobromine (2) and theophylline (3) [19]. Theobromine has little stimulant action, but has more diuretic activity and also muscle relaxant properties. Theophylline also has low stimulant action and is an effective diuretic, but it relaxes smooth muscle better than caffeine or theobromine [37]. Excessive intake of these alkaloids may cause undesired side effects and give rise to symptoms such as tremor, tachycardia and gastro-intestinal difficulties [9, 13].

Trigonelline (7) is a pyridine derivative present in green seeds to the extent of 0.25-1% and is known to contribute indirectly to the formation of appreciated flavor products including furans, pyrazine, alkyl-pyridines and pyrroles during coffee roasting [1, 2]. Demethylation of trigonelline during roasting generates nicotinic acid (9), a water soluble vitamin B also known as niacin. Coffee is a significant source of this vitamin in the diet since nicotinic acid produced during coffee roasting is highly bioavailable in the beverage [12]. Trigonelline appears to have anti-invasive properties against cancer cells [2].

Sucrose (8) which is the most abundant simple carbohydrate in coffee, acts as an aroma precursor during roasting because it generates several classes of compounds such as furans, aldehydes and carboxylic acids that will affect the flavor of beverage [24].

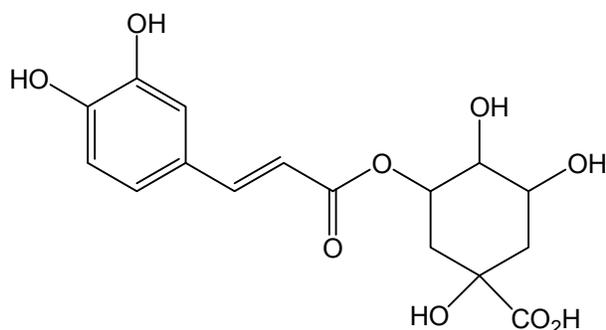
Chlorogenic acid (**4**) is a plant phenolic found in a number of edible plants, with higher concentrations in broccoli, potatoes and coffee beans [25, 26]. Chlorogenic acid increases bitterness after degradation into phenol derivatives such as caffeic acid (**6**) [1]. There are also reports that chlorogenic acid is responsible for a substantial part of the antioxidant effect of coffee [14]. Figure 1 shows the structures of the principal coffee compounds [21, 37].



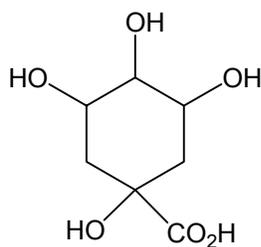
1 Caffeine, R₁ = R₂ =CH₃

2 Theobromine, R₁ = H, R₂=CH₃

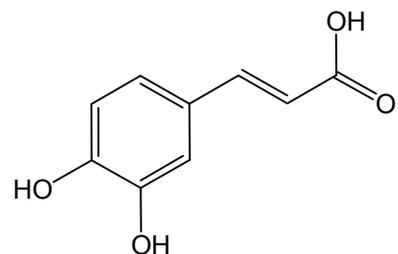
3 Theophylline, R₁ = CH₃, R₂ =H



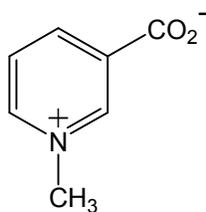
4 Chlorogenic acid



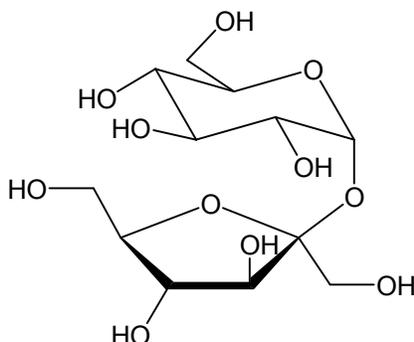
5 Quinic acid



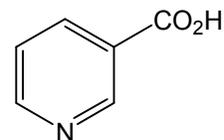
6 Caffeic acid



7 Trigonelline



8 Sucrose



9 Nicotinic acid

Figure.1. Structures of the principal coffee compounds.

The contents of caffeine, trigonelline, chlorogenic acid and sucrose in commercial coffee and tea may be highly influenced by coffee and tea species, variety, geographical origin and processing conditions which in turn affect cup quality [1, 7]. Therefore, determination of these components both before and after processing should be a useful tool for quality control of raw materials and for monitoring of coffee and tea processing conditions.

1.3. Caffeine: Occurrence and Consumption

Caffeine is a naturally occurring stimulant found in the leaves, seeds, or fruits of over sixty plants around the world. Caffeine exists in coffee bean, tea leaf, kola nut and cocoa bean. Because of its use by large number of people in different societies, it is the most widely used psychoactive substance in the world [34]. Due to its stimulant properties, caffeine is used around the world in any of its many forms such as coffee, tea, soft drinks and chocolate. Table1 displays the amount of caffeine in some food products [21].

Coffee has both the highest and the most variable caffeine content among dietary products containing caffeine [21]. It is suggested that values differ from 30-175 mg caffeine per cup (150 mL) of coffee [14, 21] and the standard value has been suggested to be 85 mg per cup made from ground roasted coffee [21]. The caffeine content of coffee and tea depends on the variety of coffee bean or tea leaf [1, 18], the brewing method and the length of brewing or steeping time [21]. Brewed coffee has more caffeine than instant coffee and, espresso has more caffeine (500 mg) than brewed coffee. Espresso is made by forcing hot pressurized water through finely ground, dark-roast beans [22]. Because it is brewed with less water, it contains more caffeine than regular coffee.

In soft drinks, caffeine is both a natural and an added ingredient. About 5% of the caffeine in Colas and pepper-flavored soft drinks is obtained naturally from cola nuts; the remaining 95% is added. Caffeine-free soft drinks contain virtually no caffeine and account only for 5% of all of the soft drink market [21].

Table1. Caffeine contents of some food products [21, 35]

Food/ Beverage	Volume or weight	Average caffeine content (mg)
Coffee, brewed	150 mL	85
Coffee, instant	150 mL	60
Coffee, brewed, decaffeinated	150 mL	3
Tea, brewed	150 mL	41
Tea, instant	150 mL	28
Regular colas	180 mL	15-24
Diet colas	180 mL	13-29
Chocolate bar	28 g	20
Dark chocolate, semi sweet	28 g	20
Milk chocolate candies	28 g	5

Numerous prescription and non-prescription drugs contain caffeine [13]. Caffeine increases the effectiveness of aspirin and other painkillers, justifying its addition to headache and pain-relief remedies, diet pills, diuretics and other stimulants [13, 21]. Typical caffeine-containing prescription drugs contain 30-100 mg caffeine per tablet or capsule. Nonprescription drugs contain a wide range of caffeine, 15-200 mg per tablet or capsule, depending on the type of product and brand involved [21].

1.4. Caffeine and Health

Caffeine is best known for its stimulant, or 'wake-up' effect. Once a person consumes caffeine, it is readily absorbed by the body and carried around in the bloodstream, where its level peaks about one hour after consumption. Absorbed caffeine is readily distributed throughout the entire body. It passes across the blood-brain barrier, through the placenta into amniotic fluid and the foetus, and into breast milk [32]. Caffeine mildly stimulates the nervous and cardiovascular systems. It affects the brain and results in elevated mood, decreased fatigue and increased attentiveness, probably aiding a person to think more

clearly and work harder [34]. It also increases the heart rate, blood flow, respiratory rate and metabolic rate for several hours. When taken before bed time, caffeine can interfere with getting sleep or staying asleep [32, 34].

Exactly how caffeine will affect an individual, and for how long, depends on many factors including the amount of caffeine ingested, whether one is male or female, one's height and weight, one's age and whether one is pregnant or smoker [32, 34]. Some people are more sensitive to the effects of caffeine than others. With frequent use, tolerance to many of the effects of caffeine may also develop.

Caffeine can be mildly addictive. Even when moderate amounts of it are withdrawn from 18 to 24 h, one may feel symptoms such as headache, fatigue, irritability, depression and poor concentration. The symptoms peak within 24 to 48 h and progressively decrease over the course of a week [32, 34]. To minimize withdrawal symptoms, experts recommend reducing caffeine intake gradually.

Current research on how caffeine affects a variety of health issues is summarized below. Most experts agree that moderate use of caffeine is not likely to cause any health problems.

Some studies have focused on the effects of caffeine on heart health. Moderate caffeine consumption does not appear to adversely affect cardiovascular health. Clinical studies have shown that single doses of caffeine which are less than 450 mg do not increase the frequency or severity of cardiac arrhythmia in healthy persons, patients with ischaemic heart disease or those with serious ventricular ectopia [14, 32].

Caffeine appears to increase the excretion of calcium, a mineral needed for healthy bones. Calcium is particularly important to prevent osteoporosis, a bone disease characterized by loss of bone strength and seen especially in older women. Moderate caffeine intake does not seem to cause a problem with calcium as long as one is consuming the recommended amount [23].

In the past there have been concerns that the caffeine in coffee may cause cancer. Research has shown that caffeine in coffee does not cause breast or intestinal cancer. Epidemiological studies on the carcinogenicity of caffeine as present in coffee have consistently shown that caffeine is not associated with cancer development at several tissue and organ sites. However, not enough research has been done to determine if caffeine in coffee is involved in urinary bladder or pancreatic cancer. Taken in moderation, it is unlikely that caffeine will cause cancer [32].

Evidences suggest that, at levels over 500 mg per day, caffeine may delay conception. Caffeine consumption has been associated with alteration of hormone levels (e.g. oestradiol), with tubal disease or endometriosis, with altered tubal transport time, and with reduced viability of the fertilized ovum. Moderate consumption does not appear to be of concern to women trying to get pregnant. Moderate consumption is also important for healthy pregnancy. Excessive intake has been associated with miscarriages and low birth weight babies [32].

Because children have developing nervous systems, it is important to moderate their caffeine consumption. For children, major sources of caffeine include soft drinks and chocolate [32].

Caffeine may be useful as part of a weight control program because it increases the rate at which the body burns calories for three or more hours after being consumed. Caffeine's ability to improve physical performance is well known among well trained athletes [34].

1.5. Methods of Determination of Caffeine

Several techniques have been reported for the individual quantification of caffeine as well as simultaneous determination of caffeine with different analytes in pharmaceutical preparations [9, 13, 27], biological fluids [19] and foodstuffs [2, 3, 9, 10, 12].

One of the earliest methods of caffeine determination is based up on ultraviolet (UV) spectrophotometry. Caffeine absorbs in the ultra violet region at 276 nm [13]. Although

spectrophotometry is a fast and simple method, it is not possible to determine caffeine directly in coffee beans by the conventional UV absorption due to the spectral overlap [3]. On the other hand, the derivative spectrophotometer is relatively easy; however, it requires larger concentration of caffeine in the samples [3].

The more recent methods of caffeine determination are based on the high performance liquid chromatography (HPLC) combined with several detection methods like UV, mass and infrared spectrometry [2, 12]. However, most of these reported procedures involve relatively long retention times or require lengthy sample pretreatments.

There are also other techniques reported for caffeine determination such as Fourier Transform Infrared spectroscopy [12], Ion chromatography [9], liquid chromatography coupled to mass spectrometry [2] and so on.

Nuclear magnetic resonance (NMR) spectroscopy is probably one of the most versatile analytical tools available, and has become the technique of choice for biological fluids [28, 29] and polymer tests [30], and for pharmaceutical analysis. The latter includes the determination of impurities, the contents of drugs and the characterization of isomeric drug mixtures [41].

Generally, NMR becomes quantitative NMR (qNMR) whenever it is applied as a quantitative analytical tool. In principle, qNMR is amenable to all NMR-sensitive nuclei and unrestricted in dimensions. Quantitative proton NMR (qHNMR) is the most commonly used technique in the analysis of foods, pharmaceuticals, natural products, etc. Because the second most important studied organic NMR nucleus (^{13}C) is less sensitive (1.6% of ^1H sensitivity for an equal number of nuclei) and low natural abundance (1.1%), it is difficult to obtain quantitative information by quantitative ^{13}C NMR technique, especially for small natural product samples [41].

Besides qualitative information, NMR can provide quantitative information about the sample since the intensity (or the area) of a sample is directly proportional to the number of nuclei producing the signal. The precision of the integrals determines the accuracy of

quantification, which depends on the noise level of the spectrum, the line shape, quality of shimming and phase-, baseline- and drift corrections [41].

For simple compositional analysis, integration of the spectrum or selected spectral region is performed, followed by adjustment of the integrated intensities to reflect the number of protons giving rise to the integrated signals. The individual integrated intensities are summed and then expressed as a percent of summed integrations, which represents the molar composition of the mixture (mole%). If an absolute determination of the principal component of a complex mixture is required, it is necessary to develop a weight-percent – quantitative assay. This procedure would involve obtaining a weight (mg) of a sample of the crude mixture, adding a precise quantity of a known internal standard, obtaining the solution qHNMR spectrum of the sample plus internal standard and calculating the actual weight of the desired component of the crude mixture.

The main advantage of using NMR for quantitative analysis is that it is simple and straightforward. In most applications, the sample only has to be dissolved in a deuterated solvent followed by measurement of NMR spectrum. Ideally, every signal in the NMR spectrum has the same response factor concerning the number of magnetically equivalent nuclei that constitute the signal, which make the calculation simple [30, 41]. Other advantages of NMR are its nondestructive nature and selectivity, together with the fact that it reveals structural information about previously known contaminants that may be present.

Thin-layer chromatography (TLC) is a very old method of analysis that has been well proven in practice. For more than thirty years, it has occupied a prominent position, especially in qualitative investigations. With the development of modern pre-coated layers and the introduction of partially or completely automated equipments for the various stages of operation of TLC, not only are highly accurate quantitative determinations now possible, but also the requirements that the work should comply with good manufacturing practice (GMP)/ good laboratory practice (GLP) guidelines can be fulfilled [6, 39].

Thin layer chromatography can be applied in different areas of analysis: pharmaceuticals and drugs, clinical chemistry, forensic chemistry, biochemistry, food analysis, environmental analysis, natural products chemistry, synthetic organic chemistry and other areas [6, 39].

In situ scanning of TLC plates, employing optical instrumentation, has been extensively developed over the past decade and is now considered essential for both the accurate identification of the spot position and the precise quantitative estimation of its content [6, 43]. In most instruments, the plate surface can be examined employing reflected light, transmitted light or fluorescent light. In addition the incident light may be absorbed, diffusely scattered or transmitted through the plate. The normal procedure is to measure the light scattered, absorbed, reflected or generated by fluorescence from the spot and compares it electronically with the light from a part of the plate where no sample has passed (e.g. the channel between the spots). Single and double beam instruments are available for scanning densitometry analysis [6, 39, 43].

The sensitivity of a scanning densitometer is governed by a number of factors that are determined by the basic instrument design and, in particular, the quality of the optical system. The plate surface is scanned through a slit and the major factor affecting the overall sensitivity is the slit height to spot diameter ratio. Although the slit dimensions are usually selectable, as the spots along the plate will be of different size, it is not possible to adjust the slit dimensions to the same optimum size for scanning the whole of the plate [39].

Band-shaped starting zones give the best possible separation efficiency attainable with the selected TLC system. For quantitative analysis, the Linomat spraying method is claimed by the manufacturer to give further advantages [39]. The first is that as the distribution of the substance is uniform over the whole width of the band, densitometric evaluation can be performed by measurement of aliquots. This enables the best possible accuracy to be achieved. The second is facilitation of operation: if the Linomat spraying method is used for calibration purposes, different volumes of the same solution should be applied instead of the same volumes of solutions of different concentrations. This

considerably reduces the amount of work required. The standard addition method can be performed with the Linomat by over spraying. In some cases, a pre-chromatographic *in situ* derivatization by over spraying the applied samples with reagent solution can be performed.

2. Objectives of the Study

2.1. General Objective

The main aim of the work is to develop and validate rapid quantitative proton NMR and thin-layer chromatographic methods for the quantification of caffeine that could be applied routinely to different types of raw and processed coffee beans as well as tea and beverages

2.2. Specific Objectives

- Employing qHNMR and quantitative TLC for the determination of caffeine in different coffee, tea and soft drink samples.
- Baseline separation of caffeine from all other matrix compounds that are extracted during sample preparation and a specific detection for the accurate results.
- Developing accurate and rapid method of extracting caffeine from raw and processed coffee and other products.
- Optimizing all steps of the analytical process with respect to practicality and convenience ensuring broad applicability.

3. Materials and Methods

3.1. Standards and Chemicals

Theophylline (minimum 99%), caffeine (99%) were purchased from Sigma and magnesium oxide from BDH. Analytical grade chloroform (99.0-99.4%) and methanol (99.5%) from Riedel-de Haën, acetone (99.99%) from Fisher Chemicals, deuterated water (99.8%) from Sigma were also used.

3.2. Coffee, Tea, Drug and Soft Drink Samples

Fourteen different samples were used in this study. Three green coffee bean samples were obtained from three different private shops at different times. From the two green coffee bean samples, some amount was roasted and all samples were ground. One commercial, ground, roasted coffee sample ('Abyssinia coffee') was obtained from a supermarket. Two locally brewed coffee samples ('Abol' and 'Bereka') were supplied by Professor Ermias Dagne. Two commercially brewed samples were taken at different times from Cordial Cafeteria in front of the Science Faculty, Addis Ababa University. One commercial tea named 'Gumaro Tea' was obtained from a supermarket. Two soft drinks, Coca-cola and Pepsi-cola, were bought from a cafeteria. Two aspirin tablet brands, Epharm and Bayer, were obtained from a pharmacy.

3.3. Preparation of Standards

For TLC analysis, two standards were prepared. Standard solution I: 10 mg of caffeine was dissolved in 10 mL of chloroform (1 $\mu\text{g}/\mu\text{L}$) and used as a stock solution. Standard solution II: standard solution I was diluted with chloroform 1:10 (100 $\text{ng}/\mu\text{L}$). Both solutions were stored at 6 °C.

For qHNMR analysis, one standard solution was prepared: 10 mg of theophylline was dissolved in 25 mL acetone (0.4 mg/mL) and stored at 6 °C.

3.4. Sample Preparation

Local coffee brewing: The brewing procedure was done according to the Ethiopian traditional coffee brewing method. 20 g of green coffee beans was roasted, ground and transferred to 'Jebena' containing 300 mL hot water. The mixture was boiled and finally 175 mL 'Abol' was obtained, of which 50 mL was taken for analysis. To the residue, 150 mL hot water was added and boiled again. 50 mL of the supernatant (called 'Bereka') was taken for analysis.

Raw coffee, roasted coffee, tea and drug samples were extracted in duplicate according to the modification of the method of Ky *et al.* [1]. 100 mg of dried and ground sample was mixed with 100 mg of magnesium oxide and 1 mL distilled water in a flask. The mixture was heated on steam bath for 20 min. To the residue, 5 mL chloroform was added and extracted by sonication for 15 min and anhydrous sodium sulphate was added to it. The mixture was filtered through filter paper (Whatman No. 1) and washed three times with 5 mL chloroform. The final volume was adjusted to 15 mL with chloroform.

Brewed coffee and soft drink samples (5 mL of each) were taken and 100 mg of magnesium oxide was added to each sample. The mixture was freeze dried (Christ Alpha 1-2) for 6 h. To each residue, 5 mL chloroform was added and extracted by sonication for 15 min, then anhydrous sodium sulphate was added. The mixture was filtered through filter paper (Whatman No. 1) and washed three times with 5 mL chloroform. The final volume was adjusted to 15 mL with chloroform.

For TLC analysis, the filtrate was directly used as a test solution. For qHNMR analysis, the filtrate was dried in a rotavapor, a known amount of theophylline reference solution (0.4 mg/mL in acetone) was added to it and dried. The mixture was then dissolved with 0.6 mL deuterated water and transferred to NMR tube.

3.5. Water Content

To express the amount of caffeine on dry weight basis (dwb), the water contents of all ground coffee and tea samples were determined by overnight oven drying of the samples at 105°C [2, 7].

3.6. Quantitative Proton NMR Analysis

The proton NMR experiments were performed on a Bruker ACQ 400 AVANCE spectrometer operating at 400 MHz equipped with a 5 mm multinuclear probe and running Topspin 1.3 software at 298 K. The spectra of 500 μL samples were recorded in neutral D_2O media. Typical acquisition parameters for ^1H NMR experiments were as follows: acquisition time 4.0 s, spectral width 8278.146 Hz, relaxation delay 1 s and number of scans 4.

The spectral data processing included Fourier transformation (FT) of the free induction decay (FID) data using MestRe-C software, phase correction (performed using automatic phase correction procedure after FT) and baseline correction for the entire spectral range. In all instances, the baseline was additionally corrected over the integral regions. Areas of peaks were determined by electronic integration of expanded regions of selected resonances.

3.6.1. Calibration Curve Preparation

To a known amount of theophylline reference solution (0.4 mg/mL), 0.4, 0.8, 1.2, 1.6, 2.0 mg/mL of caffeine solutions were added and the mixtures were dried using a rotary evaporator, dissolved in 0.6 mL D_2O and analyzed by the proposed method. The calibration curve was generated by plotting the peak area of the analyte relative to an internal standard as a function of the weighted amounts of its concentration [30].

3.6.2. Precision and Accuracy

The intra- and inter-day variation of the method for the determination of caffeine was investigated at three different concentration levels of 0.4, 0.8, 1.6 mg/mL and was expressed in terms of percent relative standard deviation (%R. S. D).

3.6.3. Recovery Studies

Three pre-analyzed samples were spiked with extra 0.4, 0.8, 1.6 mg/mL of the standard caffeine and the mixture were reanalyzed. This was done to check for the recovery of caffeine at lower, middle and upper levels in the samples.

3.7. Thin-layer Chromatographic Analysis

Samples and standards were applied on pre-coated TLC plates (aluminium sheets, silica gel 60 F₂₅₄, 10x10 cm, 0.25 mm layer thickness, Merck) as 8 mm bands, 14 mm distance between tracks using the 'spray on' technique. A Camag Linomat 5 (Switzerland) was used for sample application. TLC plates were developed with chloroform: methanol (10:1 v/v) over a distance of 50 mm from the lower edge of the plate using Camag twin trough chamber (Switzerland), saturated for 10 minutes with the mobile phase. The developed plates were then dried in air for 5 minutes.

The quantification of caffeine was performed by densitometric evaluation in absorption mode at 276 nm using deuterium and tungsten lamps. A Camag TLC scanner 3 equipped with deuterium and tungsten lamps, set at 276 nm in absorption mode, was used for scanning the plates. The size of the scanning slit was adjusted to 6.0x0.45 mm and the scanning speed to 20 mm/s at a data resolution of 100 μm /step. The peak heights and areas of chromatograms were determined using winCATS Software.

3.7.1. Calibration Curve Preparation

From the stock solution II (100 ng/ μ L) 2, 5, 8, 11, 14, 16 μ L were spotted on the TLC plate to obtain concentrations of 200, 500, 800, 1100, 1400, 1600 ng spot⁻¹ of caffeine, respectively. The data of peak areas were plotted against concentration to generate the calibration curve using winCATS software. The concentrations of standards were selected in such a way that they could cover a wide range of concentrations detected in different samples.

3.7.2. Precision

Repeatability of the sample application and measurement of peak areas were carried out using three replicates of the same spot (6 μ g) of caffeine and were expressed in terms of percent relative standard deviation (%R. S. D). The intra- and inter-day variation for the determination of caffeine was carried out at three concentration levels of 400, 600 and 800 ng spot⁻¹.

3.7.3. Recovery Studies

A pre-analyzed sample was spiked with extra 301, 602 and 910 ng of the standard caffeine and the mixtures were reanalyzed. This was done to check for the recovery efficiency of the method at different concentration levels in samples.

3.7.4. Specificity

The specificity of the method was ascertained by analyzing the standard caffeine and extract. The spot for caffeine in the sample was confirmed by comparing the R_f value and spectrum of the spot with that of the standard. The peak purity of caffeine was assessed by comparing the spectra at three different levels: peak start, peak apex and peak end positions of the spot.

For raw coffee, roasted coffee and tea samples, the percent caffeine content was determined. For brewed coffees and soft drinks, the amount of caffeine (in mg) per cup/bottle of the samples was determined.

4. Results and Discussion

4.1. Quantitative ^1H NMR Analysis of Caffeine

4.1.1. Qualitative Analysis

Typically, the ^1H NMR spectra of theophylline (**3**) and caffeine (**1**) can be divided into regions containing resonances characteristic of the functional groups present in their structures. Figure 2 shows the proton NMR spectra of theophylline, caffeine and a sample containing both compounds, plotted in a chemical shift range of 2.0 to 8.0 ppm. The corresponding ^1H NMR spectra shown in Figure 2 can be divided into two regions containing signals arising from the aromatic protons, 7.0-8.0 ppm, and the methyl group protons linked to nitrogen atoms, 3.0-4.5 ppm. The spectrum of theophylline (**3**) exhibits two characteristic, sharp singlet peaks at δ 3.24 and 3.43 that belong to the methyl groups linked to the nitrogen atoms as well as a singlet peak at δ 7.89 corresponding to the =C-H proton. The peak corresponding to the proton linked to nitrogen is very weak because of its exchange with water protons. The spectrum of caffeine (**1**) consists of three sharp singlets at δ 3.22, 3.40 and 3.83 due to the three methyl groups connected to the nitrogen atoms as well as a singlet peak at δ 7.78 corresponding to the =C-H proton.

It should be noted that, in all spectra in Figure 2, a sharp singlet peak is observed at δ 4.70 corresponding to water. The methyl signals of theophylline (**3**) at δ 3.24 and 3.43 show a small shift of about 0.02 ppm (downfield) relative to the methyl signals of caffeine (**1**) and consequently, result in some overlapping.

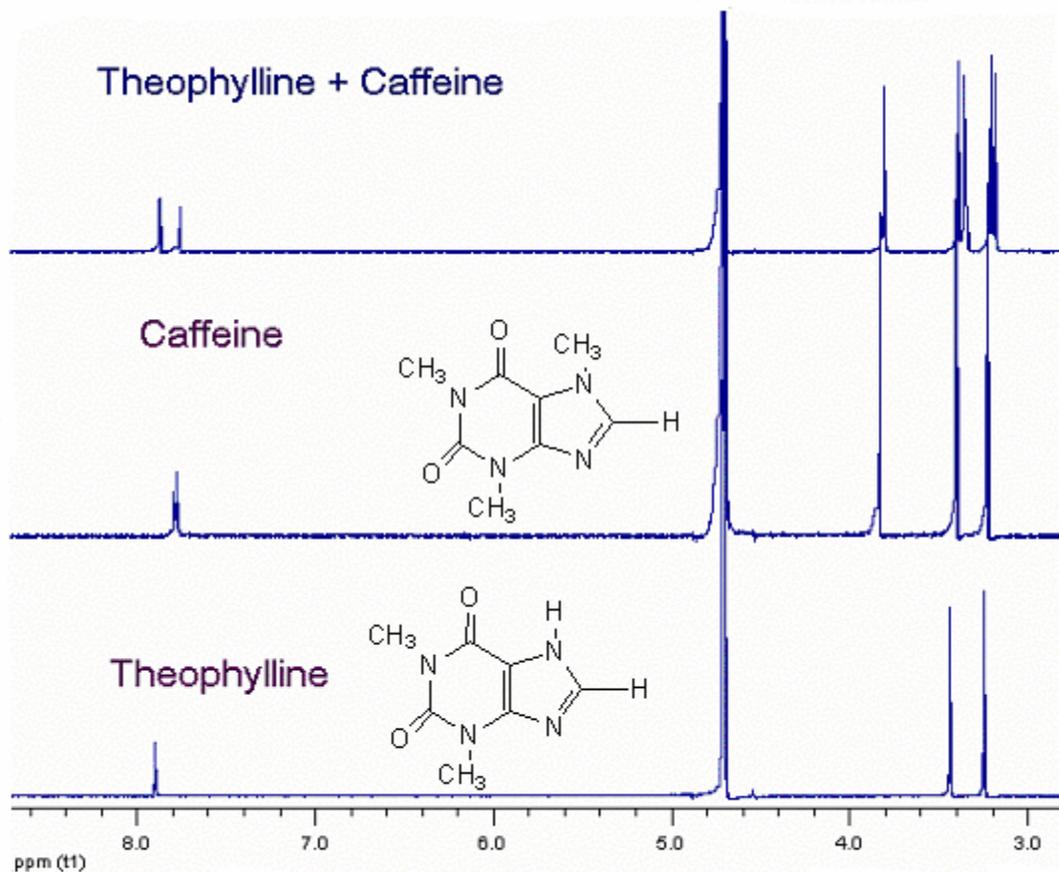


Figure 2. Proton NMR spectra of theophylline, caffeine and a sample containing theophylline and caffeine.

4.1.2. Quantitative Analysis

For quantification purpose, a chemical shift position was selected. Three criteria were used for this selection: (i) minimum overlap with other lines of the spectrum, (ii) minimum number of lines corresponding to spin-spin coupling, in order to obtain the best signal-to-noise ratio and precision, and (iii) absence of exchange phenomena. The region 2 in Figure 3 best fulfills these criteria. This spectral region generally shows well-resolved NMR peaks displaying one-to-one correspondence between theophylline and caffeine.

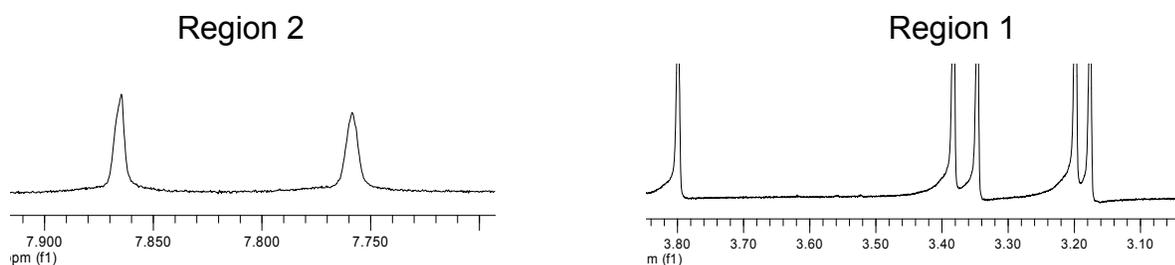


Figure 3. ^1H NMR of a sample containing both theophylline and caffeine standards classified in to two regions: signals arising from protons of methyl groups attached to nitrogen atoms (Region 1) and aromatic protons (Region 2).

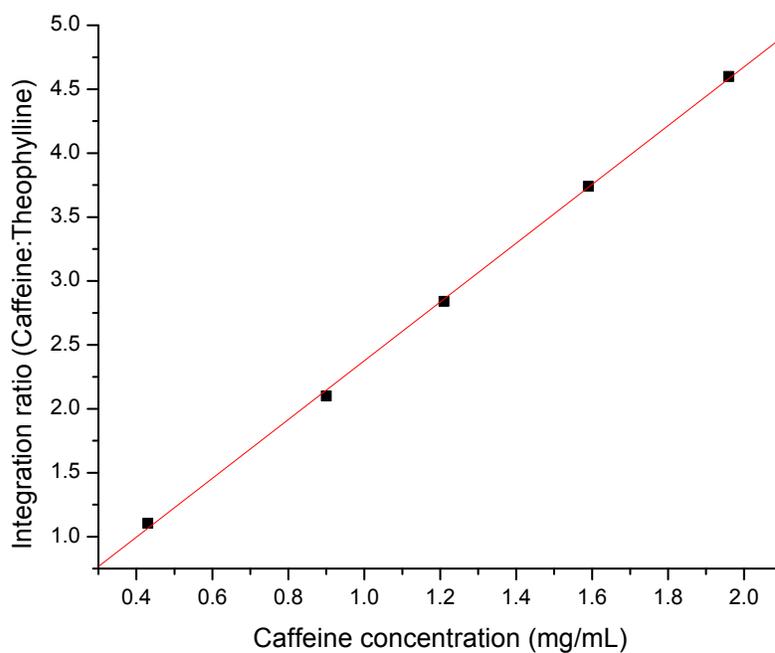
The quantification by NMR spectroscopy needs to take into consideration the ratio of signals belonging to different compounds with respect to those of an internal standard. This procedure can be carried out on the basis of either peak integration (area) or peak intensity (height). Throughout this work, integration of selected signals was used for quantitative analyses. The integration ratio gives the mole ratio of caffeine and theophylline in the NMR tube. The mass of theophylline is known, so the following formula can be used to calculate the mass of caffeine in the NMR tube [28].

$$W_C = \frac{W_T I_C MW_C}{I_T MW_T}$$

Where W_C is measured weight of caffeine (mg), W_T is the weight of theophylline (mg), MW_C the molecular weight of caffeine, MW_T the molecular weight of theophylline, I_C and I_T are the integrals of selected resonances of caffeine and theophylline, respectively.

4.1.3. Calibration Curves

The peak area of the analyte relative to internal standard as a function of the weighted amount of its concentration allowed the creation of calibration plots (Figure 4). The mean value (\pm S.D) of slope and intercept were 2.299 ± 0.03 and 0.077 ± 0.04 , respectively. No significant difference was observed in the slopes of standard curves. The response linearity was ensured by the coefficient factor 0.9997. In the range of 0.4 - 2.0 mg/mL caffeine concentration, a good linearity was observed, which is very convenient for the determination of caffeine in coffee, tea and other products.



Linear Regression equation:

$$I = A + B * C$$

I = Integration ratio (caffeine: theophylline)

A = Intercept

B = Slope

C = Concentration of caffeine

Parameter	Value	Error	
A	0.07667	0.04235	
B	2.29895	0.03187	
R	SD	N	P
0.99971	0.03786	5	<0.0001

Figure 4. Calibration curve for 0.4 - 2.0 mg/mL caffeine concentration

4.1.4. Method Validation

The intra- and inter-day accuracy and precision of the method were investigated by analyzing three different concentrations of caffeine. For the intra-day assay, each analysis was performed three times and for the inter-day assay, the same determination was performed for three days (three times each day). The percent R. S. D ranged from 1.26 to 2.33 for intra-day, and from 0.76 to 2.39 for inter-day variation (Table 2).

Table 2. Intra-and inter-day accuracy and precision of the q¹H NMR method.

Spiked amount (mg)	Intra-day (n = 3)		Inter-day (n = 3)	
	Amount found (mg) (Mean ± S.D)	%R.S.D	Amount found (mg) (Mean ± S.D)	%R.S.D
0.40	0.43 ± 0.01	2.33	0.44 ± 0.003	0.76
0.80	0.90 ± 0.02	2.22	0.87 ± 0.02	2.39
1.60	1.59 ± 0.02	1.26	1.60 ± 0.01	0.81

The accuracy of the quantification was assessed in a recovery study (Table 3). Three samples with pre-determined caffeine content were individually spiked with three different amounts of caffeine to reach a final content in the lower, middle and upper range of the pseudo-linear calibration. The proposed method, when used for estimation of caffeine, afforded recovery of 97 to 108% as shown in Table 3.

Table 3. Results of Recoveries (n = 3)

Predetermined amount in sample (mg)	Amount added(mg)	Theoretical amount (mg)	Amount found (Mean ± S.D in mg)	Recovery (%)
0.80	0.40	1.20	1.16 ± 0.01	97
0.90	0.80	1.70	1.69 ± 0.08	99
0.70	1.6	2.30	2.49 ± 0.06	108

4.1.5. Application to Samples

In order to evaluate the applicability of the method, samples of green and roasted coffees, brewed coffees, tea and soft drinks were analyzed. The contents of caffeine in these samples, obtained by quantitative TLC and qHNMR methods, are shown in Table 6 and Table 7.

The peaks used for integration were selected at 7.72 - 7.78 and 7.82 - 7.88 ppm for caffeine and theophylline, respectively (Figure 5). The spectrum displays that at neutral P^H , for sample measurements, the selected peaks are well resolved and no overlapping occurs.

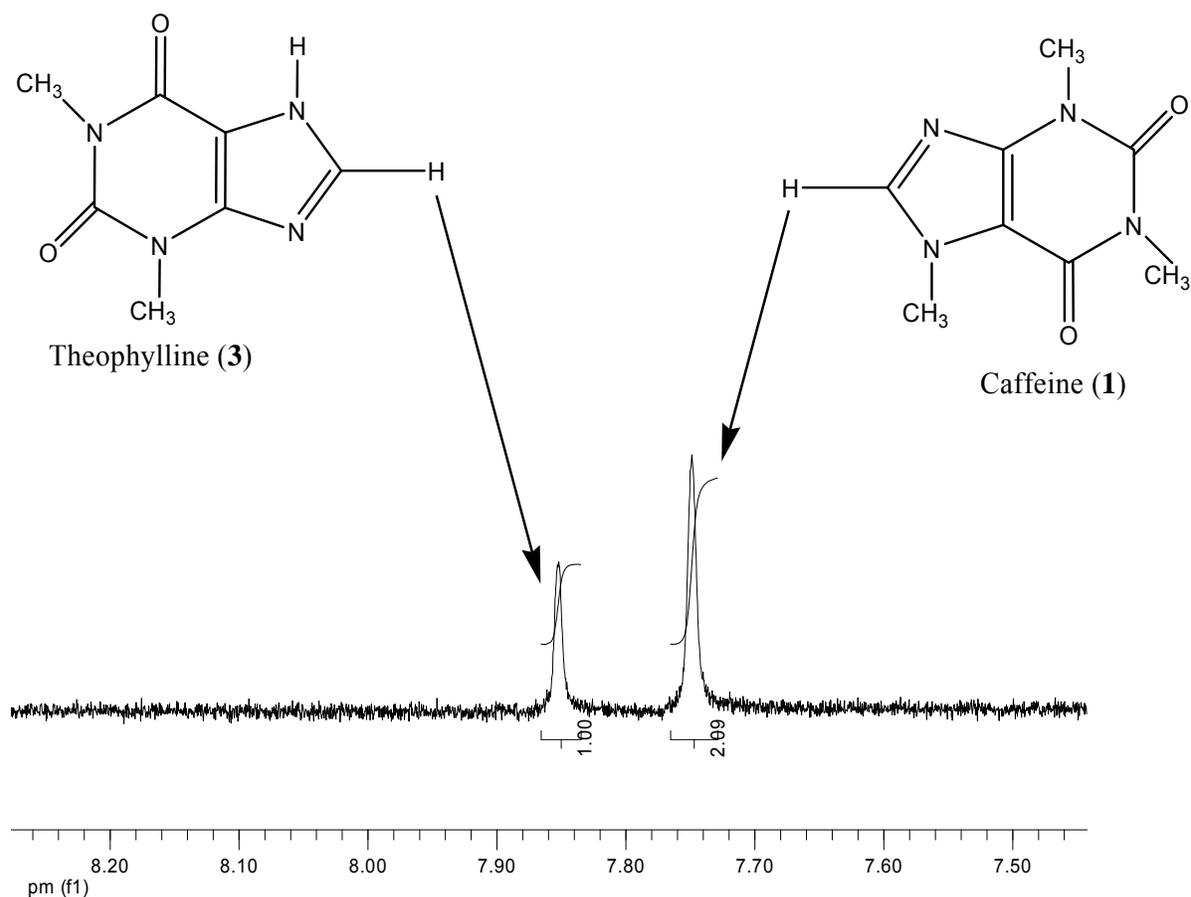


Figure 5. The 1H NMR spectrum of a typical coffee extract sample containing 0.9 mg of caffeine and 0.4 mg of reference theophylline.

Caffeine values ranged from 0.8 to 1.0 % (dwb) in green (A, B, C) and roasted (D, E, F) coffee samples. The % caffeine content (dwb) of a commercial tea brand 'Gumaro Tea' was found to be 2.1%. Caffeine amounts in two brewed samples, taken from the same cafeteria at different times, were 86 and 103 mg per 150 mL of coffee. This variation might be due to either the difference in coffee source or the difference in the length of brewing time [21]. For the two locally brewed samples, 'Abol' and 'Bereka', the values were found to be 36 and 12 mg per 150 mL, respectively. The two common cola soft drinks in Ethiopia, Coca-cola and Pepsi-cola, contained 19 and 15 mg caffeine per bottle (300 mL), respectively. All of these values are in agreement with caffeine contents reported elsewhere [2, 3, 21].

4.2. Quantitative TLC Analysis of Caffeine

4.2.1. Development of the Optimum Mobile Phase

The TLC procedure was optimized with a view to quantify caffeine in the coffee, tea and soft drink extracts. The mobile phase consisting of chloroform and methanol (10:1 v/v) gave a good resolution, and a sharp and well-defined peak at $R_f=0.64$ for caffeine in the standard as well as in the extracts (Figure 6 and Figure 7). Well-defined spots were obtained when the developing chamber was saturated for 10 minutes at room temperature. This enables us to accurately quantify caffeine in dilute solutions.

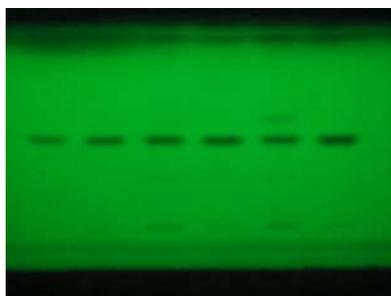


Figure 6. TLC of caffeine standard and two extracts developed with chloroform, methanol (10:1). Left to right: Tracks 1, 2, 4, 6 represent 200, 500, 800, 1100 ng caffeine standard respectively; tracks 3 and 5 represent 500 ng tea and 450 ng coffee extracts, respectively

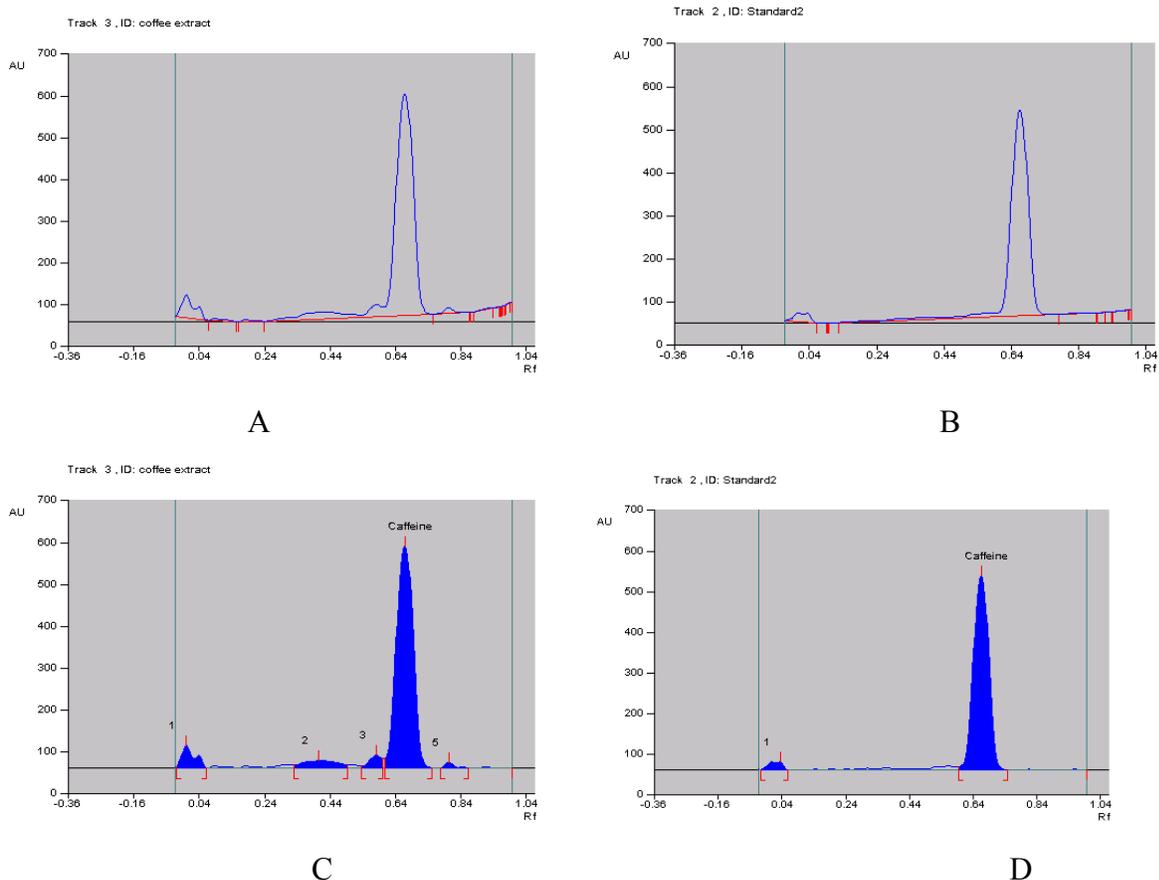
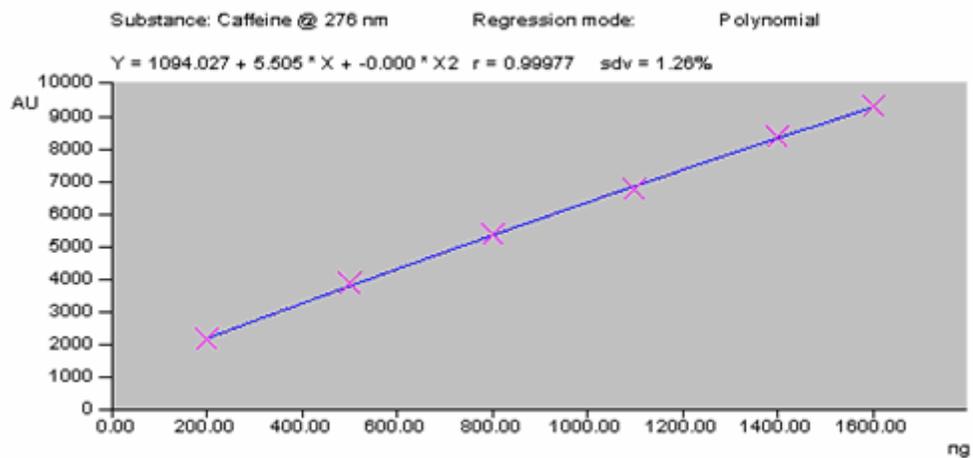


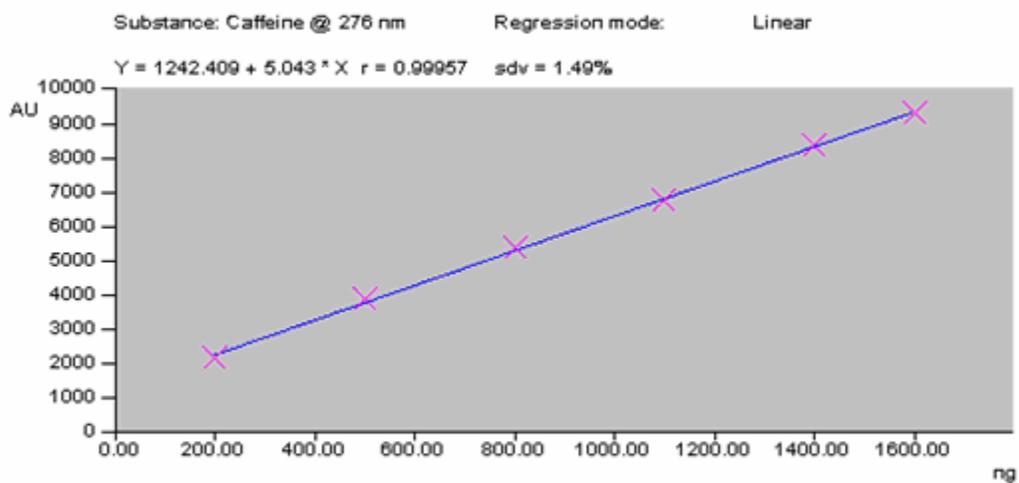
Figure 7. Detection of caffeine in a coffee sample (A, C) and as reference (B, D) after chromatography with mobile phase; absorption measurement at 276 nm.

4.2.2. Calibration Curves

The developed TLC method for estimation of caffeine showed a good correlation coefficient ($r = 0.9996$) in the concentration range of 200 to 1600 ng spot⁻¹ with respect to the peak area. The mean value (\pm S.D) of slope and intercept were 5.043 ± 0.01 and 1242.409 ± 0.01 , respectively (Figure 8F). Figure 9 displays the three dimensional image of the calibration samples at 276 nm. No significant difference was observed in the slopes of standard curves.



E



F

Figure 8. Calibration curves for caffeine standards using polynomial (E) and linear (F) regression modes.

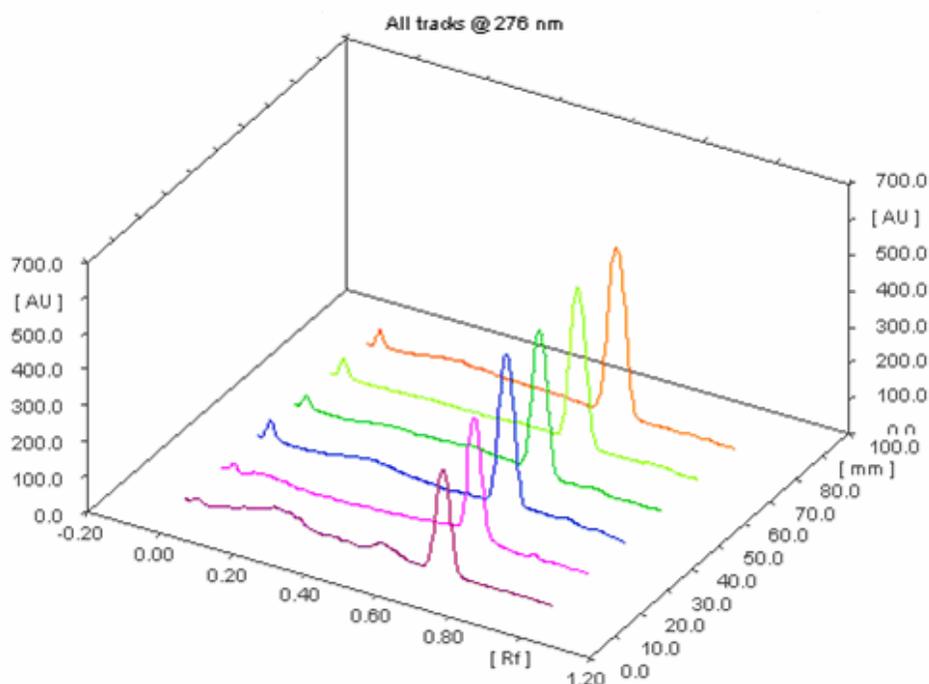


Figure 9. The Three-dimensional image of the calibration spots for caffeine.

4.2.3. Method Validation

The %R. S. D. for repeatability of sample application ($6.000 \mu\text{g spot}^{-1}$) and measurement of peak areas were found to be 4.36 and 1.97%, respectively. The measurement of the peak areas at three different concentration levels showed low values of %R. S. D for intra- and inter-day variation, which suggested an excellent precision of the method (Table 4).

Table 4. Intra- and Inter-day precision of TLC method (n=3)

Amount (ng spot^{-1})	Intra-day precision		Inter-day precision	
	Mean area from Camag	%R.S.D	Mean area from Camag	%R.S.D
400	4793	1.23	4974	2.78
600	6263	1.83	6419	2.76
800	7433	2.62	7736	3.52

The accuracy of the quantification was assessed in a recovery study (Table 5). A sample with known caffeine content was diluted and individually spiked with three different amounts of caffeine to reach a final content in the lower, middle and upper range of the pseudo-linear calibration. The proposed method, when used for estimation of caffeine, afforded recovery of 99 to 103% as shown in Table 5.

Table 5. Results of recovery study.

Caffeine in sample (ng)	Spiked amount (ng)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)
440	301	741	765	103
440	602	1042	1045	100
440	910	1350	1341	99

The peak purity of caffeine was assessed by comparing the spectra at peak start, peak apex and peak end position of the spot. Good correlation ($r = 0.9999$) was obtained between the standard and the sample overlain spectra of caffeine (Figure 10).

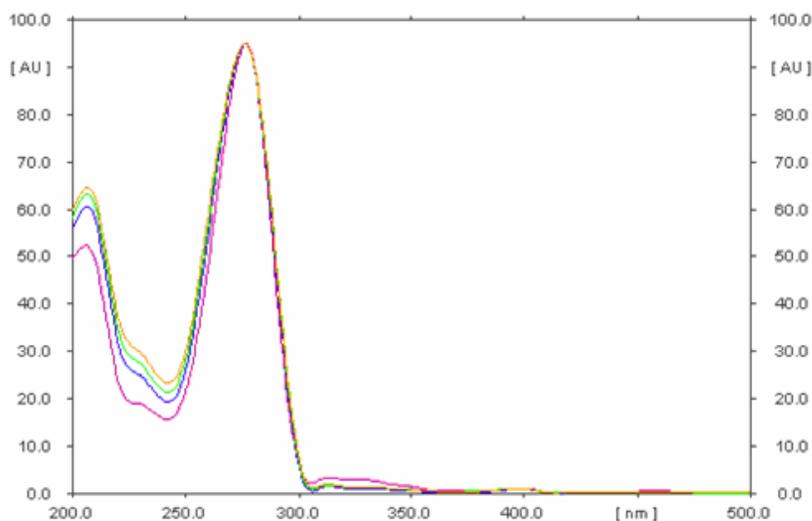
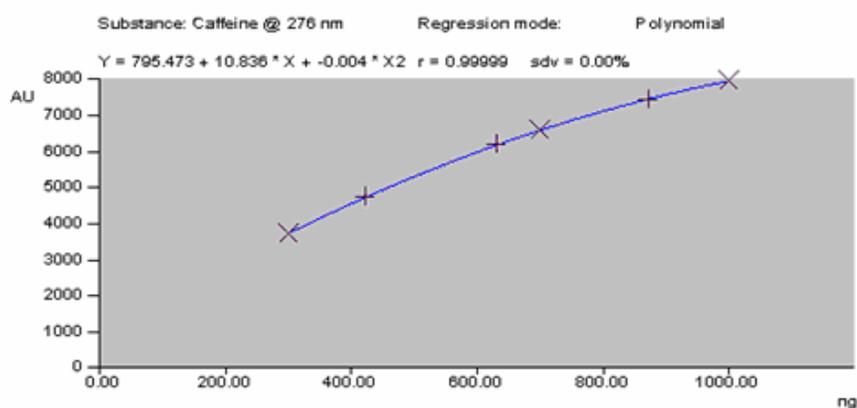


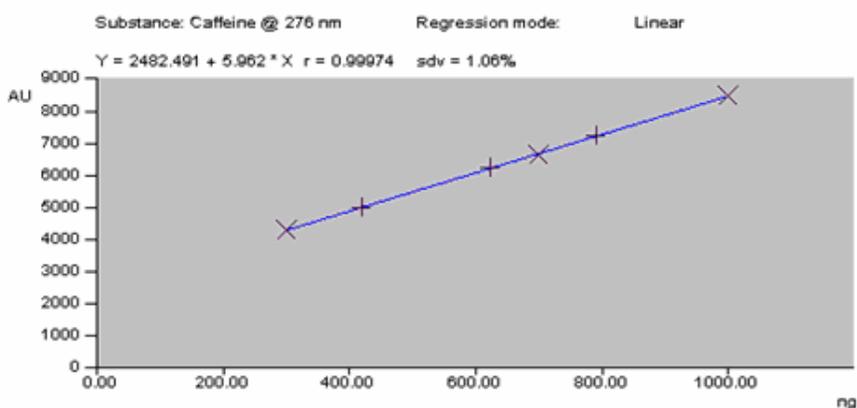
Figure 10. Spectral comparison of standard caffeine and coffee extract.

4.2.4. Application to Samples

Samples of coffee, tea and soft drinks analyzed by quantitative ^1H NMR method were again analyzed by quantitative TLC method. The results are summarized in Table 6 and Table 7. A single spot at $R_f = 0.64$ was observed in the chromatogram of the caffeine isolated from the extract along with other components. There was no interference in analysis from other components present in the extracts. These components appear in the chromatogram at significantly different R_f values as indicated in Figure 7. The curves for estimation of caffeine in three samples on one TLC plate are indicated Figure 11.



G



H

Figure 11. Quantification of caffeine in three samples on one TLC plate using polynomial (G) and linear (H) regression modes.

In green (A, B, C) and roasted (D, E, F) coffee samples, caffeine values ranged from 0.8 to 1.1% (dwb). In the two commercially brewed coffees taken from the same source at different times, the values were found to be 80 and 100 mg per 150 mL, respectively. For locally brewed coffees, 'Abol' and 'Bereka', caffeine contents were 39 and 15 per 150 mL, respectively. In Coca-cola and Pepsi-cola, the amounts of caffeine were found to be 22 and 15 mg per bottle (300 mL), respectively. Again these results are in agreement with those reported elsewhere [2, 3, 21].

Table 6. Percent caffeine contents of green coffee, roasted coffee and tea samples.

Sample	Percent caffeine content (dwb) obtained by	
	Quantitative TLC	qHNMR
Green coffee		
A	1.1	1.0
B	1.0	0.9
C	1.0	0.8
Ground roasted coffee		
D	0.9	0.8
E ^a	0.9	0.9
F	0.8	0.9
Tea		
G ^b	1.9	2.1

^a Commercial ground, roasted coffee sample named 'Abyssinia Coffee'

^b Commercial tea produced in Ethiopia named 'Gumaro Tea'.

Table 7. Caffeine contents of brewed coffees and soft drinks.

Sample	Volume of sample (mL)	Caffeine content (mg) obtained by	
		Quantitative TLC	qHNMR
Commercial brewed coffee ^c	150	80	86
Commercial brewed coffee ^c	150	100	103
Abol	150	39	36
Bereka	150	15	12
Pepsi-cola	300	15	15
Coca-cola	300	22	19

^c Samples taken from the same cafeteria at different times.

Finally, we looked at the comparison of the quantitative TLC and qHNMR methods for the quantification of caffeine in samples we studied. Results are compared in Table 6 and Table 7. With one exception, good agreements of the results were obtained in both methods. A slightly higher variation was observed in one of the commercially brewed coffee samples (Table 7). Furthermore, we tried to determine caffeine in aspirin tablets, since there are reports suggesting the presence of caffeine in prescription and non prescription drugs. Two brands of aspirin tablets, Epharm and Bayer products, were analyzed. In both tablets no caffeine was found. In fact, caffeine was not described as an ingredient in both tablets

In general, both methods were found to be suitable, fast, simple and reliable for the estimation of caffeine in coffee beans, brewed coffees, teas and soft drinks. The TLC method has two advantages. The first is that it avoids the use of expensive deuterated solvents. It is also possible to analyze more than one sample at a time in the same plate, which considerably reduces the analysis time and the amount of work required. The qHNMR method has the advantage of confirming the chemical structure of the analyte even in the presence of impurities.

5. Conclusion and Recommendation

The developed quantitative thin-layer chromatography and proton NMR techniques are simple, rapid, accurate and precise methods for the determination of caffeine in coffee, tea and soft drink samples. Both methods showed appropriate recoveries and repeatabilities. In the TLC method the proposed mobile phase effectively resolves caffeine and thus, the method can be used for quantitative as well as qualitative analysis of caffeine in different samples. In the proton NMR method, interferences by impurities are avoided, provided they have distinct lines in the spectrum. The described techniques require minimal sample preparation, only a few analytical reagents, and readily available internal standards. The analysis results demonstrate that caffeine can be effectively resolved and quantitated in the described samples and a variety of caffeine-containing samples available in the market by the proposed methods.

Since chlorogenic acid, trigonelline and sucrose are also important components of coffee that affect cup quality, further work has to be done to develop and validate qHNMR and thin-layer chromatographic methods to determine these components, either individually or simultaneously. The application of both methods can be extended to other raw materials or products containing caffeine.

Limitations of the Study

The study is mainly concerned with development and validation of qHNMR and thin-layer chromatography methods for the determination of caffeine in coffee and other products. Most of the work was devoted to optimizing the analytical conditions for the applicability and convenience of the methods. Statistically sufficient number of samples was not analyzed. In addition coffee samples were selected, with no information about the species and geographical origin. Therefore, it requires further work in which samples could be analyzed with statistically sufficient data and with known species and geographical origin to arrive at a conclusion about the caffeine contents of the samples.

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^c Books and Journals found in our lab

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