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Determination of Caffeine in Some Sudanese Beverages by High Performance Liquid Chromatography

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Abstract: Simple High Performance Liquid Chromatographic (HPLC) method was validated for determination of the content levels caffeine in 15 different beverage samples commercially available in Sudan local markets. Shim-pack VP-ODS column was used with methanol: water (30:70)% (v/v) eluent. The detector wavelength was set at 270 nm. Linearity of the method was check from 10-100 ppm and the correlation coefficient was 0.9999. The method detection limit was 0.023 ppm and the precision was 1.25% at 40 ppm caffeine concentration.. the spiked recoveries for caffeine were 99%, 105%, 99.2%, 102% and 102% in Pepsi Cola, Coca Cola, Red Bull, Gazaltain black tea and Coffee samples respectively. The caffeine contents in tea samples ranged from 440 ppm to 473 ppm with average concentration of 458.6 ppm. The caffeine concentrations in energy drinks samples ranged from 170.6 ppm to 324 ppm with average concentration of 255.6 ppm. The coffee sample contains 252.4 ppm. The carbonated soft drinks showed caffeine content in the range of 32.4 ppm to 133.3 ppm with average concentration of 96 ppm. In addition, the concentrations of caffeine have been converted into the daily intake doses based on beverages consumption. The mean values of caffeine daily intakes were 183 mg, 101 mg, 64 mg and 38 mg through the ingestion of tea, coffee, energy drinks and soft drinks, respectively.

Key words: Caffeine, beverages, HPLC, validation

INTRODUCTION

Caffeine is a naturally occurring substance found in the leaves, seeds or fruits of over 63 plants species worldwide (Abdul Mumin et al., 2006; Nour Violeta et al., 2008; Wanyika et al., 2010; Violeta Nour et al., 2010). It is an alkaloid of methylxanthine family (Wanyika et al., 2010; Marcia et al., 2002). Caffeine chemical formula is $C_8H_{10}N_4O_2$ and its systematic name is 1,3,5-trimethylxanthine. Pure caffeine occurs as odorless, white, fleecy masses, glistening needles of powder. Its molecular weight is 194.19 g, melting point is 236°C, point at which caffeine sublimes is 178°C at atmospheric pressure, pH is 6.9 (1% solution), specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760 mm Hg at 178°C, solubility in water is 2.17%, vapor density 6.7 (Komes et al., 2009; Nour Violeta et al., 2008; Hiroshi Ashihara et al., 1996; Abdul Mumin et al., 2006). Its structural formula is as shown in Fig. 1. The widespread occurrence of caffeine in a variety of plants played a major role in the long-standing popularity of caffeine-containing products. The most important sources of caffeine are coffee, tea, guarana, cola nuts and cocoa (Nour Violeta et al., 2008; Liew Siew et al., 2001; de Azevedo et al., 2008; Violeta Nour et al., 2010). The amount of caffeine found in these products varies, the highest amounts are found in guarana (4-7%),
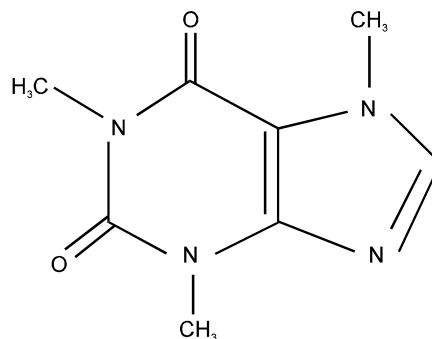

Fig. 1: Structure of caffeine

followed by tea leaves (3.5%), coffee beans (1.1-2.2%), cola nuts (1.5%) and cocoa beans (0.03%) (Komes et al., 2009).

Caffeine is added to soft drinks as a flavoring agent, it is part of the overall profile of soft drinks, which consumers enjoy for refreshment, taste and hydration. Most of the caffeine in cola drinks is added during the formulation process (Marcia et al., 2002; Nour Violeta et al., 2008; Dionex, 2007). In the case of carbonated beverages the variability occurs among brands, since most of the caffeine content in these products is added from other natural sources, i.e. less than 5% of the total present

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caffeine is from cola nuts. Cola like drinks account for 80 to 90% of the caffeine added to foods today (Violeta Nour *et al.*, 2010).

Caffeine is a pharmacologically active substance and depending on the dose, can be a mild central nervous system stimulant, improve cardiac performance, increase brain circulation, and exhibit vasodilatory and diuretic effect. It is also increase heartbeat rate, dilate blood vessels and elevate levels of free fatty acids and glucose in plasma. 1 g of caffeine leads to insomnia, nervousness, nausea, ear ringing, flashing of light derillum and tremulosness. In cases of overdosing and in combination with alcohol, narcotics and some other drugs, these compounds produce a toxic effect, sometimes with lethal outcome (Mamina and Pershin, 2002; Ben Yuhas, 2002; Wanyika *et al.*, 2010; James *et al.*, 1990; Tavallali and Sheikhaei, 2009).

Caffeine dose not accumulate in the body over the course of time and is normally excreted within several hours of consumption (Nour Violeta *et al.*, 2008).

Caffeine is considered to be a risk factor for cardiovascular diseases and may affect behaviour effects of depression and Caffeine is one of the most comprehensively studied ingredients in the food supply, with centuries of safe consumption in foods and beverages. In 1959, the United State Food and Drug Administration (USFDA) designated caffeine in cola drinks as "Generally Recognized As Safe" (GRAS). The FDA considers caffeine safe for all consumers, including children. In 1987, following extensive review, the FDA "found no evidence to show that the use of caffeine in carbonated beverages would render these products injurious to health." More than 140 countries have specifically considered the safety of caffeine and allow its use in beverages at various levels (Nour Violeta *et al.*, 2008).

Caffeine was classified as a drug of abuse by the International Olympic Committee (IOC) when present in urine at concentration levels of more than 12 µg/mL. As it is the case with any food, the composition of soft drinks is regulated by legislations. According to Directive 2000/13/EC, quinine and/or caffeine used as a flavoring in the production or preparation of a foodstuff must be mentioned by name in the list of ingredients immediately after the term "flavoring". In addition, according to Directive 2002/67/EC of 18 July 2002, drinks containing caffeine in excess of 150 mg/L must also provide a warning message on the label followed by an indication of the caffeine content such that: "High caffeine content (X mg/100 mL)".

Therefore, the determination of caffeine compounds in soft and energy drinks for assurance of food safety and quality control is mandatory (Violeta Nour *et al.*, 2010).

Several analytical techniques have been developed for the determination of Caffeine and the quality control of products containing Caffeine. For the determination of Caffeine in beverages, various analytical techniques including titrimetry, spectrophotometry, derivative spectrophotometry (G¨uzin Alpdogan *et al.*, 2002), polarography, NIR-spectroscopy, capillary electrophoresis, voltametric techniques (Ahmad *et al.*, 2005), GC (Liew Siew *et al.*, 2001) and HPLC have been reported (Nour Violeta *et al.*, 2008). Titrimetric methods suffer the disadvantage of using a large amount of sample and undergoing interference from many redox reagents with the determination. The polarographic method requires long analysis and suffers interferences from electrochemical impurities present in the food sample, whereas the enzymatic technique is generally considered to be too specialized for ordinary chemical laboratory use. Spectrophotometry is a fast and simple method for caffeine determination, but it cannot be used in samples with complex matrices because of background correction and techniques have been proposed such as thermal decomposition, direct ultraviolet irradiation alkaline treatment and an enzymatic method. Some visible spectrophotometric methods often require tedious pre-separation techniques to remove possible interferences from colored materials (G¨uzin Alpdogan *et al.*, 2002).

The most popular methods for caffeine determination in beverages are those which involve use of reversed-phase liquid chromatography. These methods have been used to determine caffeine levels in coffee, tea, and soft drinks (Michael *et al.*, 1985).

In Sudan, there is no authentic data about caffeine content in beverages; hence this present research study has been aimed to assess the compliance of the concentration levels of caffeine in some beverages available from local markets in Sudan. In this study we validate simple revered-phase HPLC method for caffeine determination in carbonated soft drinks, energy drinks, tea and coffee.

MATERIALS AND METHODS
Experimental: All reagents used in this study were of analytical or HPLC grade and all solutions were prepared by using distilled water.

Standard solution preparation: Caffeine stock standard solution of 1000 ppm was prepared by dissolving 0.1000 gram of caffeine standard (Sigma- Aldrich) in 80 mL distilled water and sonicated for 10 min. Then solution was transferred to 100 mL volumetric flask and the volume was completed to mark by distilled water. The stock solution was stored in dark places at +4°C.
Working standard solutions were prepared by suitable dilution for stock solution and they were prepared freshly.

Sample preparation: Different kinds of beverages brands including regular and diet cola, energy drinks, tea and coffee were purchased from different local

supermarkets and 15 samples were analyzed using the HPLC method. All measurements were performed in triplicate.

Soft drinks samples preparation: Once sample bottles were opened, the drinks were degassed by sonication, homogenized and filtered. Then each sample was filtered through a 0.45 μm syringe filter with a 5 mL syringe. Filtered drink sample of 2 mL were 5 times diluted in distilled water. 20 μl of each diluted sample was injected into the HPLC column. The relative peak areas were determined for three replicates of each dilute sample. Then the concentration of each dilute sample and finally the concentration of caffeine in soft drinks samples were calculated from calibration curve.

One sample of Pepsi cola, Coca cola, energy drink (Red Bull) was spiked with 20 ppm caffeine standard for recovery determination.

Tea and coffee samples preparation: 2.00 g of tea and coffee samples were weighed and put into 250 mL beakers.100 mL of boiling distilled water was added and let to stand for five minutes with stirring, the solution was cooled and filtered into conical flasks. 5 mL of the filtrate were pipetted into clean 50 mL volumetric flasks and made to the mark with the distilled water. The samples were filtered through 0.45 μm syringe filter and run in the HPLC system according to experimental conditions.

Tea and coffee samples were spiked with 20 ppm caffeine standard for recovery determination.

Instrumentation: The HPLC system used in this study was isocratic Waters HPLC, which consisted of a model 1515 isocratic pump, vacuum degasser and 2996 PDA detector (USA). The injector was a model 7725i Rheodyne injector with injection loop 20 μL. The analytical column used was Shim-pack VP-ODS with internal diameter 4.6 mm and length 250 mm

(Shimadzu Corporation, Kyoto, Japan). All chromatographic results were acquired and processed by Empower software (Waters Corporation). The experimental conditions are mentioned in Table 1.

RESULTS AND DISCUSSION
Method validation
Precision: The analytical precision of the method was assessed from the reproducibility of 6 determinations of 40 ppm caffeine solution and a relative standard deviation of 1.25% was calculated for peak area.

The retention time of caffeine was 7,347 min, with a relative standard deviation RSD = 0.5% therefore, in standard solutions, the HPLC method provides stable retention times.

Detection and quantification limits: Table 2 summarizes the method detection limit (MDL) and Method Quantification Limit (MQL). MDL was estimated as Standard Deviation (SD) of the peak area of seven injections multiplied by 3.14 (at n = 7). MQL was calculated by multiplying SD by 10 (Dionex, 2007).

Linearity: The calibration graph was generated using 20 μl injection loop. Six different concentrations of caffeine from 10 ppm to 100 ppm were analyzed according to experimental conditions. Then the calibration curve was established according to the obtained response (peak area) and the concentrations of caffeine in standard solutions. The results show a good linear relationship. The calibration data was summarized in Table 3. The calibration graph is shown in Fig. 2.
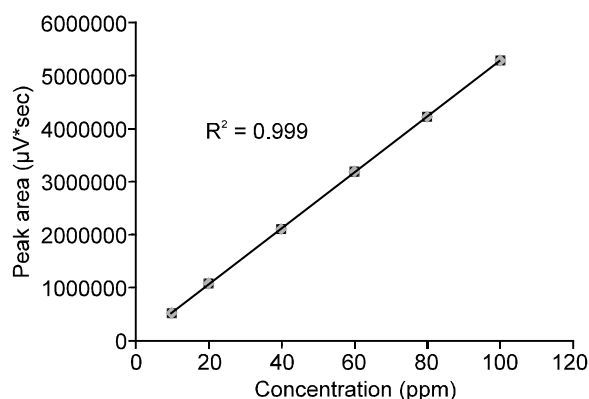

Fig. 2: Calibration curve

Table 1: Experimental conditions

Item	Conditions
Eluente	methanol: distilled water (30:70)% (v/v)
Eluente Flow Rate	1.3 mL/min
Injection volume	20 μl
Analytical column	Shim-pack VP-ODS (250x4.6) mm
Column temperature	Ambient
Detector	PDA
Detection	UV 270 nm

Table 2: Method detection and quantification limits for caffeine[a]

SD of peak area for seven replicates (ppm)	Calculated MDL (ppm)	Calculated MQL (ppm)
0.0074	0.023	0.07

[a]Standard Concentration was 1 ppm

Table 3: Regression data and statistical parameters for caffeine calibration curve

Correlation coefficient R^2	Slope (m)	Y-Intercept	Linear range (ppm)
0.9999	52726.95	10839.55	10-100

Table 4: Recoveries of caffeine from spiked beverages samples

Sample name	Amount[a] of caffeine in sample (ppm)	Amount of caffeine added (ppm)	Amount of caffeine found (ppm)	Recovery percentage (%)
Pepsi cola	18.68±0.40	20	38.47±0.80	99.0
Coca cola	18.29±0.48	20	39.30±2.00	105.0
Red bull	52.62±0.65	20	72.46±0.35	99.2
Gazaltain black tea	47.29±0.26	20	67.70±1.50	102.0
Coffee	25.24±1.60	20	45.64±0.40	102.0

[a]Average±Standard deviation (n = 3)

Table 5: Results of caffeine contents in beverage samples

Sample type	Sample name	Caffeine Con.±SD[a] (ppm or mg/L)	Caffeine Con.±SD (ppm or mg/kg)	Daily intakes[b] (mg)
Carbonated soft drinks	Coca cola	102.8±0.35	-	41.12
	Rich cola	77.0±0.50	-	30.80
	Vita cola	32.4±0.30	-	12.80
	Cola light	121.0±0.23	-	48.40
	Pepsi cola	106.2±0.77	-	42.40
	Pepsi max	133.3±0.20	-	53.20
	Pepsi diet	114.8±0.35	-	45.60
	Vitaene C	81.3±0.80	-	32.52
Energy drinks	Red bull	324.0±0.80	-	81.00
	Boom boom	273.0±0.60	-	68.25
	Tornado	170.6±0.22	-	42.65
Black tea	Gazaltein	473.0±0.26	23645.0±0.26	189.20
	Lipton	440.0±2.00	22000.0±2.00	176.00
	Mobile	463.0±1.80	23152 .0±1.80	185.20
Coffee	Ethiopian	252.4±1.60	12620.0±1.60	100.96

[a]Average ± Standard deviation (n = 3).

[b] - based on a 400 mL/day beverage consumption by a 70 kg adult (for carbonated soft drinks, black tea and coffee, 250 mL/day beverage consumption by a 70 kg adult for energy drinks)

Recovery: For recovery study one sample of known caffeine concentration form different types of beverages was spiked with 20 ppm of caffeine standard and recovery was calculated as summarized in Table 4.

As shown in Table 4 the mean recoveries of the obtained results were found to be not significantly different from the value of added caffeine concentration.

Determination of caffeine contents in beverages samples: The validated method was used to determine the concentration of caffeine in real beverages samples (carbonated soft drinks, energy drink, coffee and tea). Figure 3-6 present the chromatogram obtained for the one of the injections of caffeine standard (20 ppm) and the beverages samples, respectively.

The highest caffeine concentration in beverages samples was obtained in tea sample (black tea); this was followed by energy drinks then coffee sample. The least was obtained in carbonated soft drinks. The caffeine content levels in carbonated soft drinks, Energy drinks, tea and coffee respectively are presented in Table 5.

The caffeine contents in tea samples ranged from 440 ppm to 473 ppm with average concentration of 458.6 ppm. The caffeine concentrations in energy drink samples ranged from 170.6 ppm to 324 ppm with average concentration of 255.6 ppm. The coffee sample contains 252.4 ppm. The analyzed samples in the
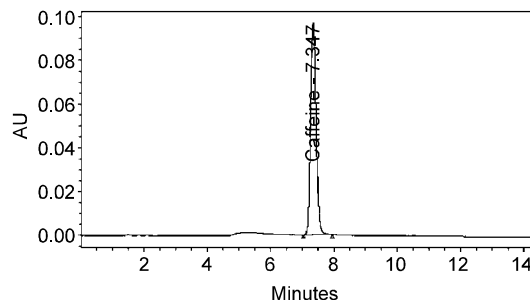

Fig. 3: Chromatogram of caffeine standard 20 ppm

carbonated soft drink group showed caffeine content in the range of 32.4 ppm to 133.3 ppm with average concentration of 96 ppm.

It was observed that the caffeine concentrations in diet carbonated soft drinks (Cola Light, Pepsi Max and Pepsi Diet) are higher than caffeine content in regular carbonated soft drinks (Coca Cola, Rich Cola, Vita Cola and Pepsi Cola) since their mean concentrations level were of 123 ppm and 79.6 ppm respectively.

All over the world, the caffeine contents in soft drinks varies according to the type of the brand, yet its average content in soft drinks is approximately 18 mg per six ounces (i.e. 100 ppm). In fact, the US Food and Drug Administration (FDA) limits the maximum caffeine amount in carbonated beverages to 6 mg/oz (200 ppm) (Violeta Nour *et al.*, 2010).
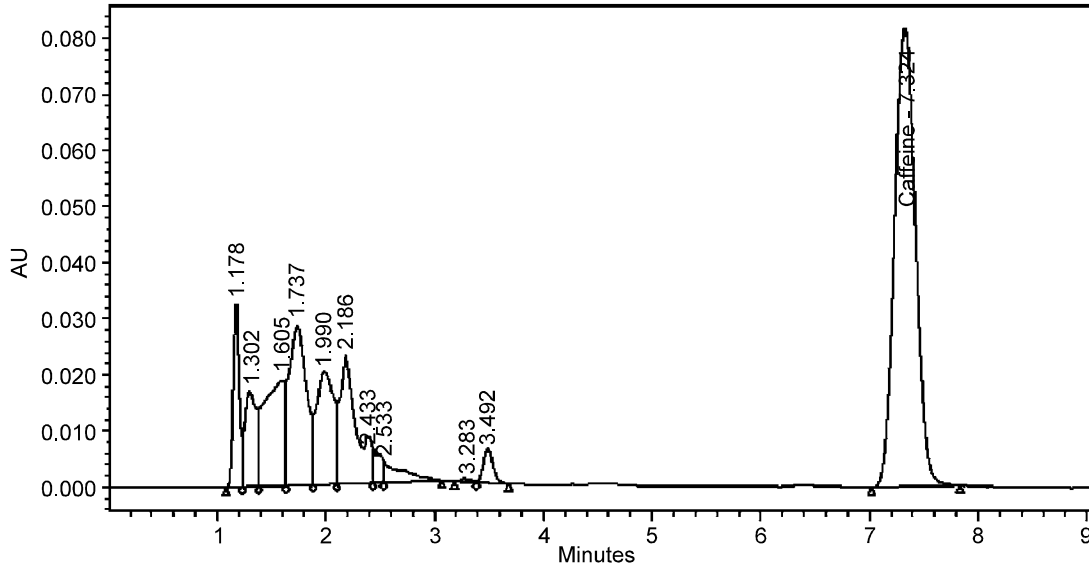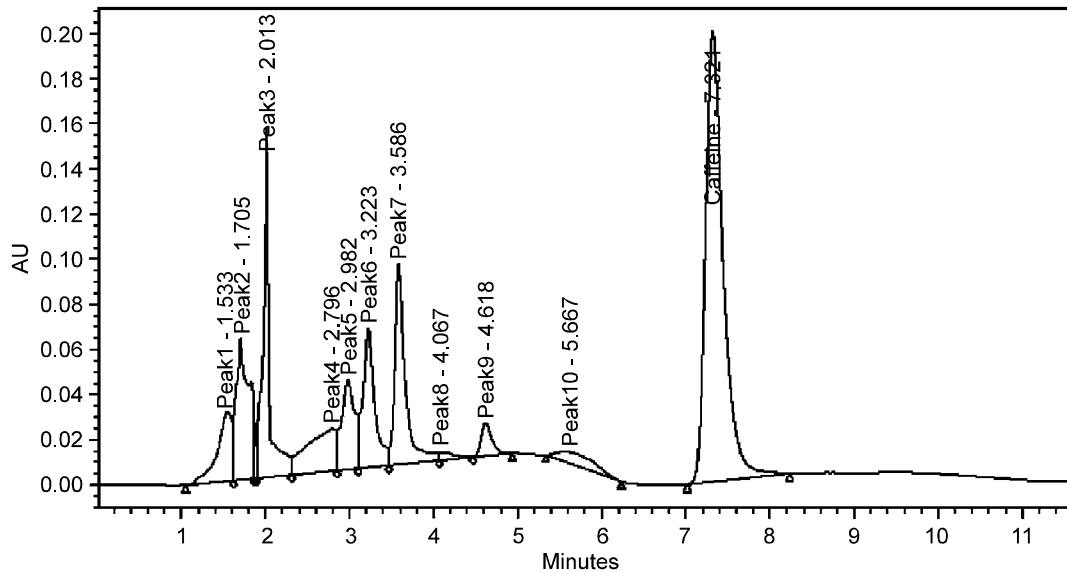
Fig. 4: Chromatogram of Coca Cola samples

Fig. 5: Chromatogram of energy drink sample (Boom Boom)

Clearly, the caffeine mean content level in the analyzed carbonated beverage samples marketed in Sudan is well below the above food industry guidelines.

In this current study it was found that tea contains higher level of caffeine than coffee. This is in agreement with previous work reported by Wanyika *et al.* (2010).

Estimation of the daily intakes of caffeine: Rationally, the daily intakes of caffeine through beverages consumption rely mainly on both the content levels of caffeine in the drinks and the amounts consumed of these drinks. Due to the fact that the amounts of the consumed foodstuffs depends on the food habits in

particular population group and in order to estimate the daily intake of caffeine in Sudan, the following assumptions have been put forward: one of the drink is consumed daily by every adult person. However, the size of the carbonated soft drinks bottles varied considerably (330, 350 and 500 mL) and energy drinks bottles (250/mL). On average, two to three cups of tea and coffee are consumed daily, the cups size are varied from (100-225 mL), hence, daily consumption rate of 400 mL of carbonated soft drinks, tea and coffee. 250 mL daily consumption rate of energy drinks. Also an average adult body weight of 70 kg were also assumed.
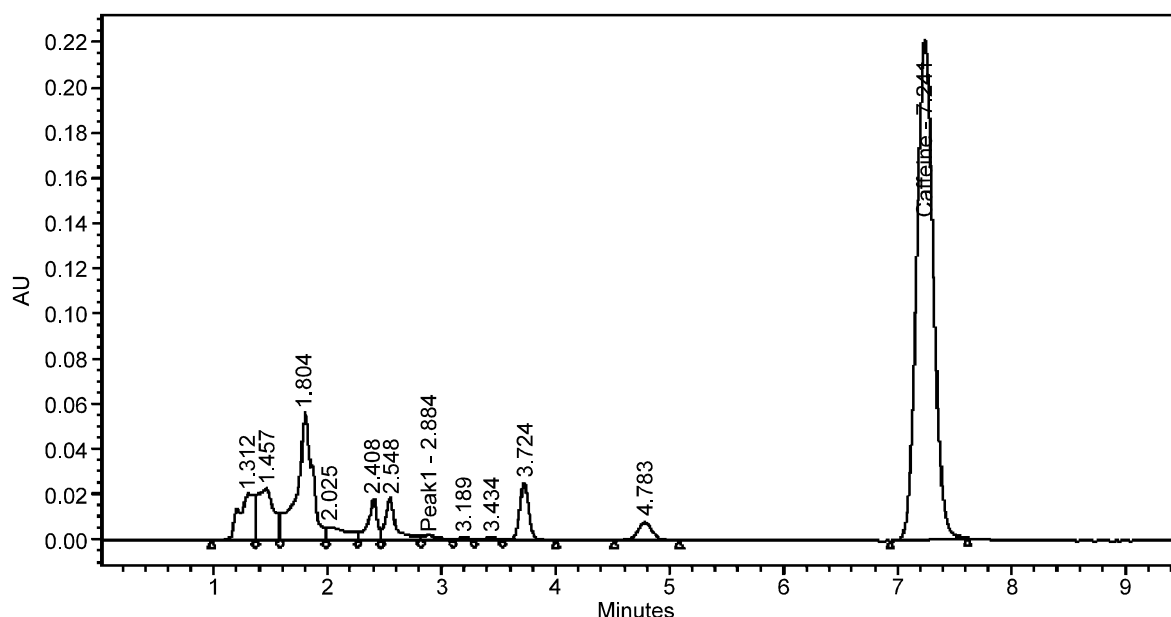
Fig. 6: Chromatogram of black tea sample (Mobile)

Table 5 illustrates the estimated values of caffeine daily intakes through the consumption of the indicated foodstuff products. As expected, the data presented in this table indicates the highest caffeine intake doses through the consumption of tea followed by coffee, energy drinks in comparison to the consumption of the carbonated drinks. The mean values of caffeine daily intakes were 183 mg, 101 mg, 64 mg and 38 mg through the ingestion of tea, coffee, energy drinks and soft drinks, respectively.

According to the International Food Information Council, the consumption level of caffeine for adults in the U.S. is approximately 200 mg per day (Ahmad *et al.*, 2005). This may indicate that the contribution from beverages in the intake of caffeine is 92%, 51%, 32% and 19% for tea, coffee, energy drinks and soft drinks, respectively.

Conclusion: The validated HPLC method for the quantification caffeine in beverages was found to be simple, precise, sensitive and accurate and allowed the obtaining of good results.

In spite of the number of drink samples analyzed is small, the data presented in this study gave a preliminarily outline about the content levels in tea, coffee, soft and energy beverages frequently consumed in Sudan.

This analytical measurement was undertaken primarily to assess the compliance of content levels of the caffeine and their daily intake doses with the permissible levels.

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