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Determination of Caffeine and some other Drugs in Cappuccino, Nescafe, Cacao, Coffee samples collected from some Libyan Markets using High Performance Liquid Chromatography (RP-HPLC)

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ABSTRACT

This study aims to determine the caffeine and detection of some other drugs (which has commonly spread and caused suicides in the recent years in El-Bieda city - Libya) in cappuccino, nescafe, cacao and coffee samples using Reversed Phase - High Performance Liquid Chromatography (RP-HPLC). In the presented study, seventeen samples were collected from some Libyan markets. A rapid, simple and reliable extraction method is developed and validated for the determination of caffeine and other drugs in the samples under study using dichloromethan, n-hexane and methanol as the extracting solvents. The method is validated over a wide linear range of 2 – 10 µg/ml with correlation coefficients being consistently greater than 0.999. The minimum caffeine level was observed in the Nesquik (Cacao), Nestle Italiana S.P.A sample (2.9129 µg/ml), while the Nestla (Nescafe), Asbania sample showed the highest caffeine content (598.5315 µg/ml). The RP-HPLC measurements indicate that the results of caffeine concentrations in samples under study are in the range of 2.9129 - 598.5315 µg/ml with an average of 221.8630 µg/ml. The measurements by HPLC methods indicate that the samples under study are free from other drugs: (olanzapine, diazepam and alprazolam). The caffeine content in all the samples analyzed in this study are within the allowable limits set by the US Food and Drugs Administration and documented values.

تقدير الكافئين وبعض المخدرات الأخرى في عينات كابتشينو، نسكافيه، كاكاو وقهوة جمعت من بعض الأسواق الليبية باستخدام كروماتوجر افيا السائل عالي الكفاءة معكوس الطور(RP-HPLC)

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الكلمات المفتاحية:
مشتقات القهوة
الكافيين
المخدرات
الاستخلاص
مذيبات
كروماتوجرافيا السائل عالي الكفاءة (*HPLC*).

الملخص

تهدف هذه الدراسة لتقدير الكافيين والكشف عن بعض المخدرات الأخرى (التي شاع انتشارها وتسببها في حالات الانتحار في السنوات الماضية في مدينة البيضاء الليبية) في عينات كابتشينو، نسكافيه، كاكاو، وقهوة باستخدام كروماتوجرافيا السائل عالي الكفاءة معكوس الطور (RP-HPLC). في هذه الدراسة تم تجميع سبعة عشر عينة (نسكافيه، كاكاو، كابتشينو، و قهوة و مشتقاتها) من بعض الأسواق الليبية. تم تطوير و التحقق من طريقة استخلاص سريعة وبسيطة و موثوق بها وذلك لتقدير الكافيين وبعض المخدرات الأخرى في العينات تحت الدراسة باستخدام داي كلورو ميثان و n-هكسان و الميثانول كمذيبات استخلاص . هذه الطريقة صالحة لمدى واسع من الخطية تتراوح من 10 - 2) ميكروجرام/مل) بمعامل ارتباط اكبر من0.999 وذلك للقياسات

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الكروماتوجرافيةHPLC)). المستوى الأقل للكافيين لوحظ في عينة نسكويك (كاكاو) 2.9129) ميكروجرام/مل) بينما عينة النسكافيه (نستله) أظهرت محتوى عالي من الكافيين 598.5315) ميكروجرام/مل). القياسات الكروماتوجرافيةRP-HPLC)) تشير إلى أن نتائج تراكيز الكافيين في العينات تحت الدراسة تتراوح من 2.9129 إلى 598.5315 ميكروجرام/مل و بمتوسط 221.8630 جزء من مليون. متوسط تركيز الكافيين في عينة (نسكافيه) نستله أكبر من تراكيز الكافيين في جميع العينات تحت الدراسة (قهوة – كاكاو). القياسات تشير إلى أن العينات تحت الدراسة خالية من المخدرات الأخرى (أولانزابين و ديازيبام و البرازولام). محتوى الكافيين في كل العينات التي تم تحليلها في هذه الدراسة تقع في الحدود المسموح بها والمشار إليها بواسطة منظمة الأغذية و الأدوية الأمريكية و القيم الأخرى الموثقة .

1. Introduction

Coffee is one of the most popular beverages that widely consumed in the world [1-2]. The level of particular compounds, including sucrose and alkaloids, therefore directly influence the final drinking quality of coffee [2]. Coffee contains a variety of pharmacologically active ingredients, and it has long been argued whether coffee drinking is beneficial or harmful for cardiovascular disease [3-5]. Caffeine is a naturally occurring alkaloid which is found in varying quantities in the leaves, seeds or fruits of over 63 plants species worldwide [6-10]. The most common sources of caffeine are coffee, cacao beans, cola nuts and tea leaves and the worldwide consumption of products derived from these natural materials means that caffeine is one of the most popular and commonly consumed drugs in the world. Caffeine is a central nervous system (CNS) stimulant of the methylxanthine class [6]. It is the world's most widely consumed psychoactive drug. Unlike many other psychoactive substances, it is legal and unregulated in nearly all parts of the world [11]. Caffeine is a bitter white crystalline xanthine' alkaloid that acts as a mild psychoactive stimulant drug. It also possesses a weak diuretic action [7-11]. In humans, caffeine acts as a central nervous system stimulant, hence it is used both recreationally and medically to reduce physical fatigue and restore mental alertness when unusual weakness or drowsiness occurs [12-13]. Caffeine is also a common ingredient of soft drinks such as cola and energy drinks where it is deliberately added as a flavoring agent and to make the drinks addictive. The concentrations of Caffeine in soft drinks samples were in the range of 0.7909 – 1.1339 µg/ml [14]. Caffeine content in soft drinks varies by brand from 10 to 50 mg of caffeine per serving [10], however the US Food and Drug Administration limits the maximum amount in carbonated beverages to 6 mg/oz [15]. In recent years, a number of these energy drinks and coffee derivatives have been introduced to the Libyan markets [14]. Their use in combination with alcohol or drugs by young adult consumers claims to improve performance and boost energy. This could further increase the health risk to consumers. Benzodiazepines are one of the most important drugs which have been used in the treatment of neuropsychological disorders such as anxiety, insomnia, agitation, depression, muscle spasms and seizures. Also, they are used in treatment of alcohol and opioid withdrawal and for inducing of sedation and amnesia in the preoperative procedures. Benzodiazepines are among the most commonly prescribed drugs [16-17]. Olanzapine is a second generation antipsychotic approved for use in treatment of schizophrenia and bipolar disorder . It was first approved for clinical use in the European Union in 1996, and has become one of the most commonly used antipsychotic drugs worldwide [18]. It is taken by mouth or by injection into a muscle [19]. A wide variety of methods have been employed including UV-Visible spectrophotmetry and High Performance Liquid Chromatography (HPLC) being the method of choice by many researchers in determining the caffeine contents of beverages [9-10, 20-24], however HPLC is a high-priced and resource consuming technique that is not typically found in most universities in developing countries. In this study, a rapid, simple and reliable extraction method is developed, based on other methods recommended by other researchers with some modifications, in order to isolate and determine caffeine and other drugs (olanzapine, diazepam and alprazolam) in tea, coffee, beverages and energy drinks or other fluids [14, 24-27]. The extraction method used is more rapid and simple compared with other extraction methods [24, 26-27]. The aim of this study is to use a rapid, and simple extraction method for the determination of caffeine and detection of some other drugs: olanzapine, diazepam and alprazolam (which has commonly spread and caused suicides have been reported in our city by the laboratories of the judicial experience center in El-bieda city - Libya) in cappuccino, nescafe, cacao and coffee samples using Reversed Phase - High Performance Liquid Chromatography (RP-HPLC).

Material and Methods

1. Chemicals and reagents:

All chemicals, analytical standards, reagents, and solvents which used in this study were analytical grade and highly pure. Caffeine was purchased from (BDH-Analor) with purity 99.2 % (for research and development use only). Also, other chemicals and solvents were used including (Dichloromethane (CH_2Cl_2) (AlphaChemika™), with purity 99.7 %; n- Hexan (SCP), with purity 95 %; Methanol (Scharlau), with purity 99.8 %) as the extracting solvents; Formic acid (Riedel-Dehaen AG Seelze Hannover), with purity 98 – 100 %); Tris (hydroxymethyl)-aminomethan BDH Laboratory Supplies; Hydrochloric acid 25% (Riedel-dehaen); HPLC-grade methanol (Fisher Chemical), with purity 99.9 % .

2. Preparation of standard solutions:

Standard caffeine solution: (BDH-Analor): A stock solution of (0.553 mg/ml) was prepared by dissolved 0.0553 g in 100 ml methanol. The working standard solution were prepared by appropriate dilution of the stock. (2, 4, 6, 8 and 10 µg/ml). Tris base (hydroxymethyl)-aminomethan: 560.57g of Tris was dissolved in 500 ml distilled water used in extraction steps. The pH of the solution was 10.34 and the pH was adjusted by drop wise addition of Hydrochloric acid acid to (pH ≈ 9). Standard Olanzapine solution: 0.2150 g (1 capsule 5 mg) in 25 ml of solution was prepared in distilled water. Working standards were prepared by appropriate dilution of the stock. (5, 10, 15, 20 and 25 µg/ml). Standard Diazepam solution: 0.11104 g (5 capsules) in 25 ml of solution was prepared in distilled water. Working standards were prepared by appropriate dilution of the stock. (2, 4, 6, 8 and 10 µg/ml). Standard Alprazolam solution: 0.6434 g (5 capsules) in 25 ml of solution was prepared in distilled water. Working standards were prepared by appropriate dilution of the stock. (5, 10, 15, 20 and 25 µg/ml).

3. Instrumentation:

The HPLC system (Thermo Series P2000 Pump) Autosampler, Series 200 UV/Vis Detector (from 190 to 1000 nm, The Series 200 Autosampler, Series 200 Analytical Pump, Series 200 Column Oven, and 20 µl loop injector. The stationary phase represents the analytical column was a Brownlee Bio C18 column of 250x4.6 mm and 5 µm particle size.

HPLC operating conditions:

Before selecting the final operating conditions, the HPLC was operated with several solvents (mobile phases) ,included buffer solutions, and choose the ratios A% and B% and change the wavelenght (λmax)…etc. the best separation conditions are the

following:

- *Mobile Phase*: A : 35% formic acid
  B : 65% methanol
- *Flow rate*: 1.5 ml/min
- *Injection*: 20 µl
- t_R: 1.89 min for caffeine, 1.28 min (olanzapine), 5.44 min (alprazolam) and 7.48 min for diazepam.
- λ_{max} : 275 nm for caffeine, 235 nm for other drugs.

4. Standard Solutions (Calibration Curve) of caffeine and other drugs :

Calibration standards in the range 2-10 µg/ml were prepared by serial dilution from the 553 µg/ml mixed standard. The figure (1a) shows the chromatograms of different concentrations of caffeine (2 - 10 µg/ml). The retention time t_R= 1.89 min. The figure (1b) shows the chromatograms of different concentrations of olanzapine (5 - 25 µg/ml). The retention time t_R= 1.28 min. The figure (1c) shows the chromatograms of different concentrations of alprazolam (5 - 25 µg/ml). The retention time t_R= 5.44 min. The figure (1d) shows the chromatograms of different concentrations of diazepam (2 - 10 µg/ml). The retention time t_R = 7.48 min. The figure (2) show the calibration curves for the standard solutions at different concentrations of (a) caffeine (2 - 10 µg/ml), (b) olanzapine (5 - 25 µg/ml), (c) alprazolam (5 - 25 µg/ml), (d) diazepam (2 - 10 µg/ml) by HPLC.

The standard linear calibration curves obtained from the standard solutions analysis presented in the figure (2). All the figures indicate that there are a good linear relationship between the peak area and concentrations of the standard solutions.

5. Sample collection:

Seventeen samples were collected from Libyan markets. Nine of these samples were coffe samples, two were cacao samples, one cappuccino and five were nescafe samples. The table (1) shows these samples.

Table 1: Samples under study collected from Libyan markets.

S. No.	Name of Samples	Type of samples	Sources
1	Bun powder	Coffee	Albyda- libya
2	Alkalij	Coffee	Zlitn city
3	Turkish	Coffee	Turkey
4	Yamen	Coffee	Alzawya- libya
5	Al karasta coffee with coriander	Coffee	Derna- Libya
6	Khaled coffee with habahan	Coffee	Benghazi - Libya
7	Bala	Coffee	Benghazi - Libya
8	Qahwatna	Coffee	Albyda- libya
9	Nwat Altamer	Coffee	Tokra- libya
10	Caffee Break	Nescafe	MC In Egypt
11	Caffee Max	Nescafe	Turkey
12	Gold	Nescafe	Poland
13	Orga Mix	Nescafe	Egypt
14	Nestla	Nescafe	Asbania
15	Cacaoo Saied	Cacao	Tunisie
16	Nesquik	Cacao	NESTLE Italiana
17	Clever	Cappuccino	Czech rebublic

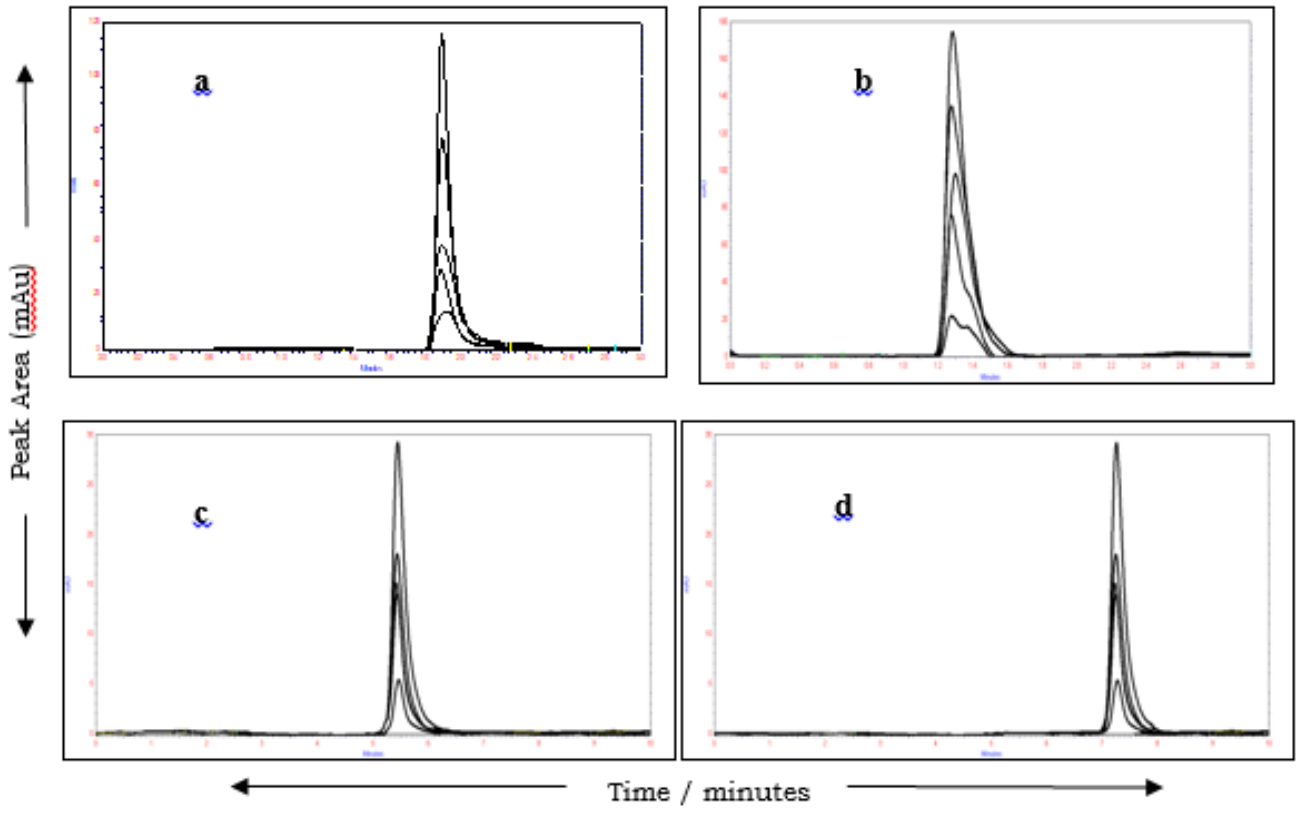

Fig. 1: The Chromatograms of different concentrations of standard soluotion caffeine (a), olanzapine (b), alprazolam (c), Diazepam (d). (**Note**: in all the 4 chromatograms : X axis = time/min ; Y axis = area (mAu)).

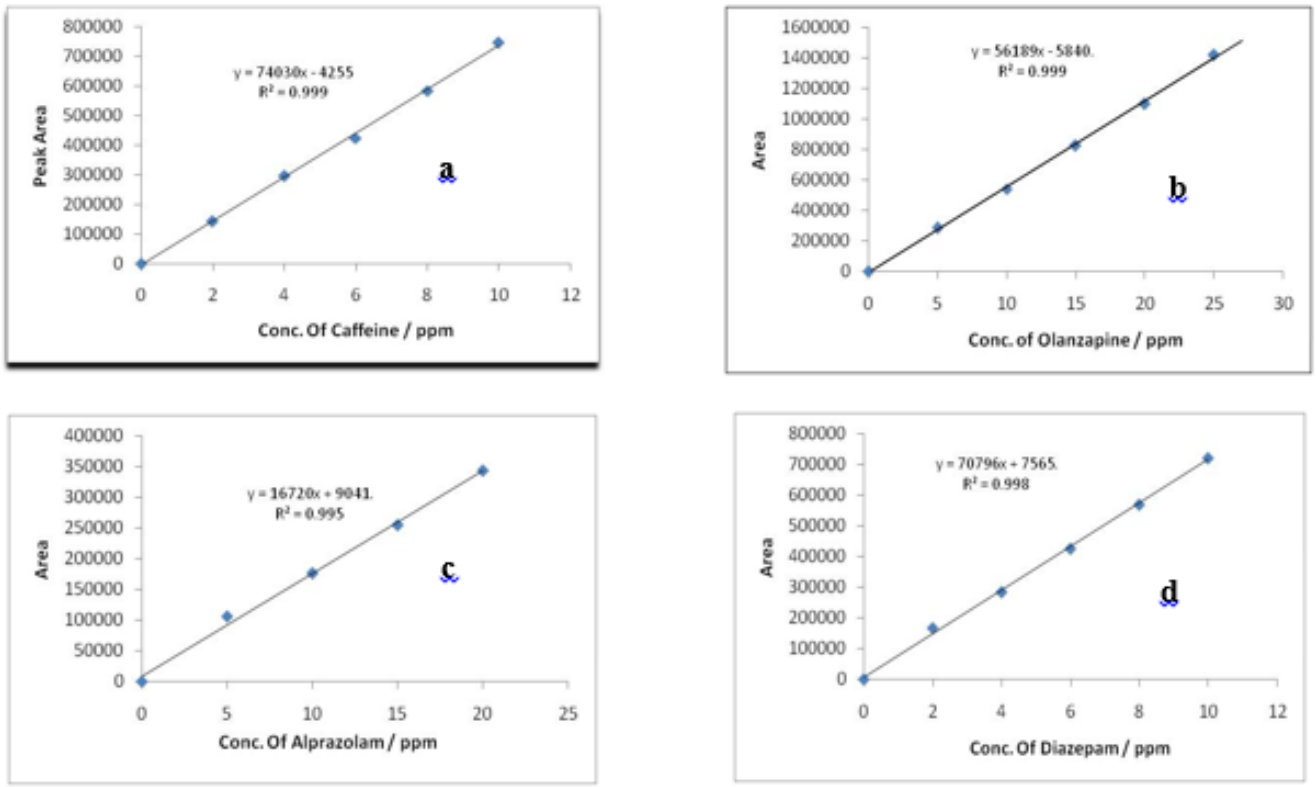

Fig. 2: Calibration curves for caffeine (a), olanzapine (b), alprazolam (c), diazepam (d), expressed on a linear scale by HPLC.

6. Sample preparation (caffeine and other drugs extraction procedure):

The extraction procedures were carried out with a slight modification based on the other study [14, 25-27].

Procedure: 10 g of the samples were weighed and dissolved in distilled water and the volumes were made up to 30 ml with distilled water (sample solution). After 15 min, the samples were filtered, 1 ml was taken and the caffeine and other drugs were extracted by the addition of 1 ml of Tris, then 8 ml of a mixture of (15 ml of dichloromethane and 35 ml of Hexane). The solutions were mixed for 15 min, and after being centrifuged at 3700 rpm for 15 min, was transferred to rotary evaporator at speed 185 and the temperature was 50 ºC . The organic phase was then evaporated by rotary at 50 ºC until dryness and reconstituted to 1 ml with methanol. A 20 µl aliquot was injected automatically into the HPLC and analyzed.

Results and Discussion

This paragraph explains the results obtained in this study, as well as highlighting the efficiency of the methods used, together with the instrumentation. The chromatograms of caffeine in all the samples illustrate only the peak of caffeine which indicate that there are percentage of caffeine in all the samples under study. The caffeine concentrations in coffee samples were in the range of 37.46135–384.211 µg/ml, while in nescafe samples were in the range of 6.8717–598.5315 µg/ml and in cacao (cappuccino) samples were in the range of 2.9129 – 189.5963 µg/ml. These results are shown in the table (2) and figure (3).

Table 2 : Concentrations of caffeine in the samples studied by HPLC method, (n=3)

S. No.	Name and Type of Samples	Conc. of Caffeine/ ppm (µg/ml)	Conc. of all other drug
S1	Coffee - Bun powder	233.8103	N.D
S2	Coffee - Alkalij	89.48207	N.D
S3	Coffee - Turkish	159.4657	N.D
S4	Coffee - Yamen	374.2011	N.D
S5	Coffee - Al karasta coffee with coriander	294.3832	N.D
S6	Coffee - Khaled coffee with habahan	37.46135	N.D
S7	Coffee - Bala	384.2110	N.D
S8	Coffee - Qahwatna	342.7807	N.D
S9	Coffee - Nwat Altamer	103.9160	N.D
S10	Nescafe - Caffee Break	393.2062	N.D
S11	Nescafe - Caffee Max	6.8717	N.D
S12	Nescafe - Gold	514.2982	N.D
S13	Nescafe - Orga Mix	39.26031	N.D
S14	Nescafe - Nestla	598.5315	N.D
S15	Cacao - Saied	7.2819	N.D
S16	Cacao - Nesquik	2.9129	N.D
S17	Cappuccino - Clever	189.5963	N.D
Average of caffeine concentration		221.863	N.D

S. No. = Sample Number; N.D = No Detected

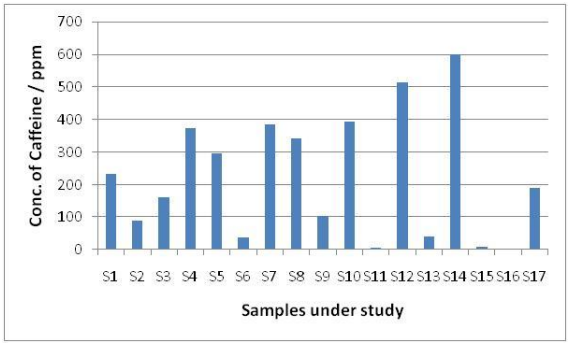

Fig. 3*:* Concentration of caffeine in the samples under study by HPLC.

These results are shown in table (2), and the figure (3) show the minimum caffeine level was observed in Cacao - Nesquik sample from NESTLE Italiana S.P.A (2.9129 µg/ml), while the highest caffeine level was observed in the Nescafe - Nestla sample from Asbania (598.5315 µg/ml). The RP-HPLC measurements indicate that the results of caffeine concentrations in the samples under study are in the range of 2.9129 - 598.5315 µg/ml with an average of 221.8630 µg/ml.

The figure (4) show the chromatogram of the caffeine in Bun powder sample (S1) as an example of the other samples under study. This figure illustrate only the peak of caffeine around t_R = 1.9 min at 275 nm and there are no peaks of the other type of drugs under study.

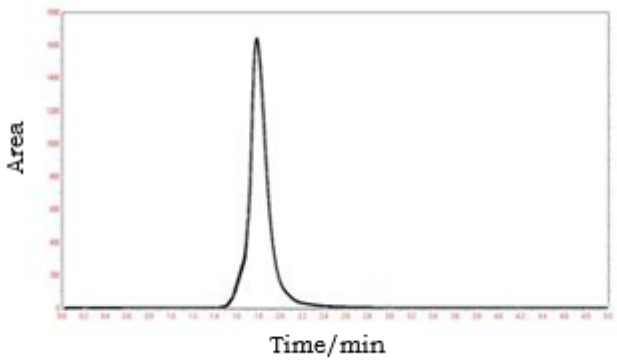

Fig. 4*:* Chromatogram of caffeine in Bun powder sample (S1) at 275 nm by HPLC.

Determination of other Drugs (Olanzapine, Diazepam and Alprazolam) by HPLC:

In this part of our study, some operating conditions of the HPLC device were changed for example wavelength at 235 nm for benzodiazepines compounds and drugs under study, also the retention times were different. The figure (5) show the chromatogram of caffeine in Bun powder sample (S1) as an example of the other samples under study that showed clear peak, while the rest did not show any clear peaks. This figure illustrate only one peak around t_R = 1.6 min at 235 nm for caffeine at these conditions and there are no peaks of the drugs under study (olanzapine, diazepam and alprazolam) compared to the standard solutions of these drugs at the same conditions.

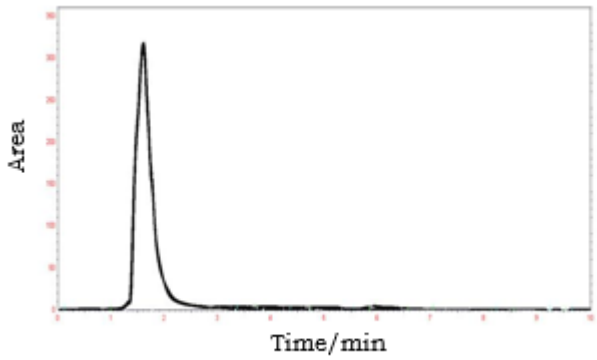

Fig. 5*:* Chromatogram of caffeine in Bun powder sample (S1) at 235 nm by HPLC.

Validation of the used methods :

There are different factors which using to validation of the analytical methods including: Linearity , Accuracy, Precision, RSD% , Recovery , LOD ,LOQ, ….etc.

1. Linearity:

Examination of calibration curves by computing a linear least-squares regression analysis on the plot of the peak area ratios and absorbances of caffeine to the external standard versus concentrations demonstrated a linear relation over the range 2 - 10 µg/ml in the case RP-HPLC (using five concentration levels) with correlation coefficients (R^2) being consistently greater than 0.999. The calibration curve (figures 2) was obtained using a microsoft office excel and it illustrate a positive linear relationship between the instrumental signal and the concentration of the caffeine standards.

2. Limit of detection (LOD) :

Is defined as the concentration of analyte required to give a signal equal to three times the standard deviation of the blank.

The LOD was calculated using the following equation [28-30]:

$$LOD = \frac{3 \cdot s_{y/x}}{b}$$

where s is the average of the standard deviation SDyx of the peak ratio (peak area of analyte/ peak area of external standard), and b is the average of the slope of a calibration curve. In the presented study, the limit of detection (LOD) value for caffeine in the samples using HPLC was 0.0189 µg/ml.

Limit of quantitation (LOQ) :

Is defined as the concentration of analyte required to give a signal equal to ten times the standard deviation of the blank. The LOD was calculated using the following equation[28-30]:

$$LOQ = \frac{10 \cdot s_{y/x}}{b}$$

The limit of quantitation (LOQ) value for caffeine in the samples in HPLC was determined to be 0.0574 µg/ml.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined in this study was more sensitive than the other reported methods [28, 30].

3. Accuracy and precision :

Accuracy is expressed as percent relative error (% R.E.). Precision is expressed as percent relative standard deviation (% RSD). In this study the Accuracy (% R.E.) = 1.862 %. Precision (% RSD) = 0.0619 %. The precision of the method (within-day variations of replicate determinations) was checked by injecting caffeine, 5 times at the LOQ level. The precision of the method, expressed as the RSD % at the LOQ level, was 0.06 % for caffeine by HPLC. This mean the calculated value of RSD% of the applied method is in an acceptable

range (less than 2%) of RSD%. For the accuracy, a standard working solution of caffeine was prepared. The prepared standards was injected 5 times as a test sample. From the respective area counts, the concentrations of the caffeine was calculated using the detector responses. The accuracy, defined in terms of % deviation of the calculated concentrations from the actual concentrations. The method of analysis is suitable for the identification and quantification of the caffeine and benzodiazepine compounds.

Conclusion

The order of caffeine concentration in the samples under study was: Nescafe>Coffee>Cappuccino>Cacaoo (by type).

The results obtained for analysis of caffeine in the samples under study using HPLC showed that there are differences in the concentrations of caffeine in these samples. The highest amount of caffeine in samples analyzed was found in Nescafe (Nestla) sample (598.5 µg/ml), while the lowest was recorded in cocaoo (Nesquik) sample (2.913 µg/ml). The extraction method used in this study provided a high efficiency. The average of the concentrations of caffeine in Nestla (Nescafe) is greater than all the other samples (coffee, cappuccino and cocaoo). The caffeine content of the samples analyzed was not found to be alarming since it correlated well with documented values. The measurements HPLC method indicated that the samples are free from other drugs under study: (olanzapine, diazepam and alprazolam). The caffeine content in all the samples analyzed in this study are within the allowable limits set by the US Food and Drugs Administration.

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