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.

Keywords:

Coffee derivatives
Caffeine
Drugs
Extraction
Solvents
RP-HPLC

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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 spectrophotometry 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 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 (CH2Cl2) (AlphaChemika™), with purity 99.7 %; n-Hexan (SCP), with purity 95 %; Methanol (Schlarau), with purity 98.9 %) as the extracting solvents; Formic acid (Riedel-Dehahen AG Seelze Hannover), with purity 98 – 100 %; Tris (hydroxymethyl)-aminomethane BDH Laboratory Supplies; Hydrochloric acid 25% (Riedel-dehahen); 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)-aminomethane: 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 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), including buffer solutions, and choose the ratios A% and B% and change the wavelength (λmax) etc. the best separation conditions are the
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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.  
- $\lambda_{max}$: 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) shows 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 coffee samples, two were cacao samples, one cappuccino and five were nescafe samples. The table (1) shows these samples.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of Samples</th>
<th>Type of samples</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bun powder</td>
<td>Coffee</td>
<td>Albyda- Libya</td>
</tr>
<tr>
<td>2</td>
<td>Alkalij</td>
<td>Coffee</td>
<td>Zlit city</td>
</tr>
<tr>
<td>3</td>
<td>Turkish</td>
<td>Coffee</td>
<td>Turkey</td>
</tr>
<tr>
<td>4</td>
<td>Alkan</td>
<td>Coffee</td>
<td>Alzawy- Libya</td>
</tr>
<tr>
<td>5</td>
<td>Khaled coffee with habahan</td>
<td>Coffee</td>
<td>Benghazi- Libya</td>
</tr>
<tr>
<td>6</td>
<td>Bala</td>
<td>Coffee</td>
<td>Benghazi- Libya</td>
</tr>
<tr>
<td>7</td>
<td>Qalhatna</td>
<td>Coffee</td>
<td>Albyda- Libya</td>
</tr>
<tr>
<td>8</td>
<td>Nwat Altamer</td>
<td>Coffee</td>
<td>Tokra- Libya</td>
</tr>
<tr>
<td>9</td>
<td>Caffee Break</td>
<td>Nescafe</td>
<td>MC In Egypt</td>
</tr>
<tr>
<td>10</td>
<td>Caffee Max</td>
<td>Nescafe</td>
<td>Turkey</td>
</tr>
<tr>
<td>11</td>
<td>Gold</td>
<td>Nescafe</td>
<td>Poland</td>
</tr>
<tr>
<td>12</td>
<td>Orga Mix</td>
<td>Nescafe</td>
<td>Egypt</td>
</tr>
<tr>
<td>13</td>
<td>Nestla</td>
<td>Nescafe</td>
<td>Asbania</td>
</tr>
<tr>
<td>14</td>
<td>Cacao Saied</td>
<td>Cacao</td>
<td>Tunisie</td>
</tr>
<tr>
<td>15</td>
<td>Nesquik</td>
<td>Cacao</td>
<td>NESTLE Italiana</td>
</tr>
<tr>
<td>16</td>
<td>Clever</td>
<td>Cappuccino</td>
<td>Czech rebublic</td>
</tr>
</tbody>
</table>

Table 1: Samples under study collected from Libyan markets.
Fig. 1: The Chromatograms of different concentrations of standard solution 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 °C . The organic phase was then evaporated by rotary at 50 °C 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)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name and Type of Samples</th>
<th>Conc. of Caffeine/ ppm (µg/ml)</th>
<th>Conc. of all other drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Coffee - Bun powder</td>
<td>233.8103</td>
<td>N.D</td>
</tr>
<tr>
<td>S2</td>
<td>Coffee - Alkalij</td>
<td>89.48207</td>
<td>N.D</td>
</tr>
<tr>
<td>S3</td>
<td>Coffee - Turkish</td>
<td>159.4657</td>
<td>N.D</td>
</tr>
<tr>
<td>S4</td>
<td>Coffee - Yamen</td>
<td>374.2011</td>
<td>N.D</td>
</tr>
<tr>
<td>S5</td>
<td>Coffee - Al karasta coffee with coriander</td>
<td>294.3832</td>
<td>N.D</td>
</tr>
<tr>
<td>S6</td>
<td>Coffee - Khaled coffee with habahan</td>
<td>37.46135</td>
<td>N.D</td>
</tr>
<tr>
<td>S7</td>
<td>Coffee - Bala</td>
<td>384.2110</td>
<td>N.D</td>
</tr>
<tr>
<td>S8</td>
<td>Coffee - Qahwata</td>
<td>342.7807</td>
<td>N.D</td>
</tr>
<tr>
<td>S9</td>
<td>Coffee - Nwat Altamer</td>
<td>103.9160</td>
<td>N.D</td>
</tr>
<tr>
<td>S10</td>
<td>Nescafe - Caffe Break</td>
<td>393.2062</td>
<td>N.D</td>
</tr>
<tr>
<td>S11</td>
<td>Nescafe - Caffe Max</td>
<td>6.8717</td>
<td>N.D</td>
</tr>
<tr>
<td>S12</td>
<td>Nescafe - Gold</td>
<td>514.2982</td>
<td>N.D</td>
</tr>
<tr>
<td>S13</td>
<td>Nescafe - Orga Mix</td>
<td>39.26031</td>
<td>N.D</td>
</tr>
<tr>
<td>S14</td>
<td>Nescafe - Nestla</td>
<td>598.5315</td>
<td>N.D</td>
</tr>
<tr>
<td>S15</td>
<td>Cacao - Saied</td>
<td>7.2619</td>
<td>N.D</td>
</tr>
<tr>
<td>S16</td>
<td>Cacao - Nesquik</td>
<td>2.9129</td>
<td>N.D</td>
</tr>
<tr>
<td>S17</td>
<td>Cappuccino - Clever</td>
<td>189.5963</td>
<td>N.D</td>
</tr>
</tbody>
</table>

Average of caffeine concentration: 221.863

S. No. = Sample Number; N.D = No Detected
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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 - Nestia 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 tR = 1.9 min at 275 nm and there are no peaks of the other type of drugs under study.

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 tR = 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.

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 (R2) 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.
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 > Cacao (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 Nescafe (Nestla) is greater than all the other samples (coffee, cappuccino and cacao). 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.

**References**


