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Determination and Characterization of Caffeine in Tea, Coffee and Soft Drinks by Solid Phase Extraction and High Performance Liquid Chromatography (SPE – HPLC)

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Abstract : Caffeine (1,3,5-trimethylxanthine), a mild addicting drug was isolated, purified and characterized from tea (black and green) and coffee. Isolation was done by liquid-liquid extraction using chloroform as an extracting solvent. Extraction was carried out in four steps such as leaching, dye removal, liquid extraction and recrystallization. Crude caffeine was purified by solid phase extraction (SPE) method. The solvent used for recrystallization were toluene and petroleum ether. The purity of caffeine was ascertained by the determination of melting temperature. Pure caffeine obtained from different samples was characterized by UV-Visible spectrophotometer, TLC, FT-IR and HPLC. In HPLC, 50mM KH_2PO_4 (pH=2), acetonitrile and methanol (40**:**8**:**2) was used as solvent as well as mobile phase. Amount of caffeine in various soft drinks (Cola) commercially available in Bangladesh was also determined by HPLC method.

Key Words: • Caffeine, • Chloroform, • SPE, • HPLC, • KH_2PO_4**,** • Acetonitrile.

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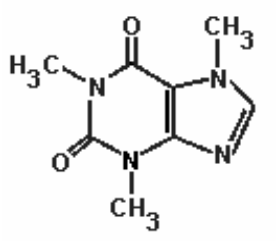
Introduction

Caffeine is a naturally occurring substance found in the leaves, seeds or fruits of over 63 plants species worldwide and is part of a group of compounds known as methylxanthines. The most commonly known sources of caffeine are coffee, cocoa beans, cola nuts and tea leaves. Caffeine is a pharmacologically active substance and depending on the dose, can be a mild central nervous system stimulant. Caffeine does not accumulate in the body over the course of time and is normally excreted within several hours of consumption [1].

Caffeine is an alkaloid of the methylxanthine family. In its pure state, it is an intensely bitter white powder. Its chemical formula is $C_8H_{10}N_4O_2$, its systematic name is 1,3,5-trimethylxanthine [2] and its chemical formula is shown below.



Structure of caffeine

Pure caffeine occurs as odorless, white, fleecy masses, glistening needles of powder. Its molecular weight is 194.19g, melting point is 236 ^{0}C, point at which caffeine sublimes is 178^{0}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 ^{0}C, solubility in water is 2.17%, vapor density 6.7 [3]

Decaffeination is a popular term in present modern world to optimize the caffeine contents in various sources. This is simply use of a solvent, which extract caffeine. For this purpose, the currently available solvents are chloroform, methyl chloride, ethyl acetate, super critical carbon dioxide etc. Methylene chloride is used to decaffeinate a high proportion of conventional teas. As a solvent, methylene chloride is highly effective, but also potentially dangerous under certain circumstances. It can cause faintness, dizziness, and headache if inhaled at high concentrations. Ethyl acetate is another compound used to extract caffeine from tea. It removes caffeine from tealeaves effectively; it can also extract other chemical components as well. Studies on green tea decaffeinated with ethyl acetate have shown the potential for up to 30% of epigallocathechin gallate (EGCG-considered to be the primary beneficial component in green tea) and other beneficial antioxidant compounds to be extracted along with the caffeine [4-6]. Ethyl acetate is also moderately toxic.

Super critical carbon dioxide (CO_2) is the fancy name for using highly pressurized carbon dioxide—the gas that adds bubbles to mineral water—to dissolve caffeine from tealeaves. The advantages of CO_2 are that it does not leave a chemical residue and it has a minimal effect on the flavor and beneficial compounds inherent to the tea [6].

Though chloroform is toxic, in laboratory purposes it is best caffeine extracting solvent and the results obtained by characterization becomes satisfactory and reproducible always [7].

In the present study, we have extracted caffeine from green tea, black tea, coffee and then characterized by melting point, λ_{max} (UV- Visible), IR absorption bands, R_f (TLC) and RT (HPLC). One of the major objectives is to develop an improved purification method based on Solid phase extraction. We have also developed an easily adaptable HPLC method for both qualitative and quantitative determination of Caffeine. In Bangladesh, there is no authentic data about caffeine content in soft drinks. So, we also determined the concentration of caffeine in various available soft drinks, especially cola drinks. The proposed method is simple, rapid and has significant advantages over spectrophotometric methods as well as other HPLC methods.

Experimental

Materials:

KH_2PO_4 (Merck, Germany); Phosphoric acid (85 %, Merck); Petroleum ether (BDH, England); Standard Caffeine (Merck, Germany); Lead Ethnoate, Chloroform, Toluene, Methanol, Acetonitrile(Merck, Germany).

Black tea (Ispahani Mirzapur tea), Coffee (Nescafe) and various brand soft drinks were collected from local market and green tea from Bangladesh Tea Research Institute (BTRI).

Apparatus:

HPLC; Model: Waters 515, with UV Detector & Vacuum filter, pH Meter (REX, Model p^{HS-25}), Visi ^{TM}-1 SPE Single Sample Processor (Supelco) and UV-Visible spectrophotometer (UV-1601,Shimadzu, Japan), Melting Electrical melting point apparatus (Electrothermal, IA9100), FT-IR (SHIMADZU, Model-IR Prestige-21), TLC (Whatman, 250µm layer, 20X20 cm).

Methods

Isolation of caffeine from tea and coffee:

5 gm solid tea was taken in 500 ml beaker and subsequently distilled water (225 ml) was added to it. The mixture was boiled for 10 min and filtered by using a Buchner Funnel. 10% lead ethnoate solution (25ml) was added with the filtrate, boiled for 5 min and filtered again. The purpose of the addition of 10% lead ethnoate is to convert any extracted tannins or other acids into anions. As electrically neutral polar molecules, the acids tend to be soluble in both water and $CHCl_3$, complicating the purification and increasing the tendency to form an emulsion in the next step. As anions they are not soluble in $CHCl_3$ and this helps to avoid an emulsion. The 10% lead ethnoate seems to cause precipitation of some substances that tend to clog the filter paper in the next step and also helps avoid formation of emulsions. A 500 ml separatory funnel was put into an iron ring on a ring stand. Pouring the tea solution in the separatory funnel and adding about 30 ml of $CHCl_3$, the solution was shaken uniformly while stopcock was opened to expel vapors. The layers were allowed to separate and the lower layer (chloroform) was collected into a 100ml beaker and the separation procedure was repeated for the second time to collect into the beaker. Anhydrous sodium sulfate was added in the beaker containing the combined extracts. The anhydrous sodium sulfate would act to remove any water and water-soluble salts that were retained in the chloroform (organic layer) or accidentally transferred during decantation. The beaker containing the extract was then heated a short period for dryness using a water bath and the temperature was controlled low enough at 70-90^{0}C to avoid caffeine decomposition. After 24 hours, white crude caffeine obtained at the bottom of the beaker. The crude caffeine obtained from the above method was purified and recrystallized by solid phase extraction method using chloroform, toluene and petroleum ether.

In case of coffee, 1 gm coffee was taken into a 500 mL beaker and subsequently 250mL hot distilled water was added. Coffee was properly dissolved in the hot distilled water. Then the whole procedure was same as that of isolation of caffeine from tea.

Solid Phase Extraction (SPE):

Commercial tea and coffee consists of many components that cause chromatographic interferences with caffeine. For this reason the sample treatment proposed consists of SPE with Sep-Pak C_{18} (500 mg) cartridges that enable separation of caffeine and remove most of the interfering components. The SPE method of Lyold et.al [8], Mottaleb et.al [9] and Cho et al.[10] was used for the extraction of caffeine. The stationary phase was activated with 3mL 50% methanol and pre-equilibrated with 3mL 1% methanol. The columns were air-dried by drawing air through them for 10 minutes. 0.5gm homogenized crude caffeine was dissolved with 100mL double distilled water and then solution (10 mL) was loaded. The adsorbed caffeine was eluted twice with 2mL

chloroform. The pure caffeine was dried subsequently and then dissolved in another solvent such as toluene and a small amount of petroleum ether was added for recrystallization. In case of HPLC analysis pure caffeine was dissolved in mobile phase. Before HPLC analysis all samples were filtered through a 0.45 μm pore size FP 30/45 CA-S filters (Schleicher and Schuell, Darmstadt, Germany) at 7 bar max. Samples (20 μL) of solutions of the samples were injected into the HPLC column.

Method of Spiking:

Different amount of black tea samples were spiked with a known amount (0.5g) of analytical standards of caffeine. The standard caffeine solution in water (20mL) was thoroughly mixed with tea solution (after leaching). To ensure homogenization, the spiked samples were shaken by using mechanical shaker for 30min. Then the solvent extraction and SPE processes were carried out as discussed before. The whole process was also carried out for only standard caffeine (0.5g) for the method validation. Unspiked tea samples were also treated similarly.

Characterization of pure Caffeine:

Different physical methods were employed to characterize the crystalline caffeine.

Determination of melting point:

The melting point of different extracted pure samples (after SPE purification) was carried out in a digital melting point apparatus (Electrothermal, IA9100). The average melting points of the samples was 235^0C.

Thin Layer Chromatography: *(Whatman, 250μm layer, 20X20 cm)*

By the purified product (crystalline caffeine) a plate was developed using chloroform as mobile phase and visualized under UV- lamp. The R_f value was measured and it was found 0.63.

IR Spectroscopy:

The IR –spectrum of extracted purified caffeine was carried out by using a FT-IR spectroscopy (SHIMADZU, Model-IR Prestige-21).

UV Spectrometry:

An UV- absorption spectrum of extracted purified crystalline caffeine was prepared at different absorbance against different wavelength using a UV-absorption spectrophotometer from SHIMADZU Corporation, Japan; Model: UV -1601. The λ_{max} was found to be 275 nm.

HPLC Optimization (calibration):

The different known concentration of caffeine in a solvent, which consisted of 50 mM potassium dihydrogen phosphate (pH=2), acetonitrile and methanol (40:8:2), which was also mobile phase. Standard solutions were injected in HPLC pump by using a syringe after setting proper flow rate (0.5mL/min), attenuation (64) and chart speed (0.5 cm/min) for which a typical pressure is about 1328-1331psi. UV detector was used at a maximum wavelength of 254nm and chromatograms were obtained with almost same retention time. Three replicates of each standard were taken and a plot of relative peak area vs. concentration made to obtain calibration curve.

Determination of caffeine in soft drinks:

Each soft drink was degassed properly by placing it in a vacuum flask and connecting the flask to a vacuum pump for 30 minutes. It was kept under vacuum until no more bubbles appear. Then each sample was filtered through a 0.45μm syringe filter with a 5mL syringe. 2 mL of filtered drink sample was 10 times diluted by using mobile phase (solvent). 20 μL of each diluted sample was injected into the column and recorded the trace. The relative peak areas were determined for three replicates of each dilute sample. Then the concentration of each dilute sample and finally the real concentration of caffeine in soft drinks samples were calculated from calibration curve. [11]

Determination of caffeine in Green Tea, Black Tea and Coffee:

1.0 mg of isolated purified caffeine was dissolved in 100mL mobile phase (solvent). Then the sample was filtered and determined by HPLC as previous section.

Reproducibility:

The reproducibility of this method was also checked by determining the percentage recovery of known amount of standard caffeine in the sample. For example if X be the actual content of caffeine in the sample and 5ppm standard caffeine was added then the content become (X+5)ppm and if the observed concentration is X', then the percentage recovery is given by

$$\% \text{ Recovery} = \frac{X'}{(X+5)} \times 100\%$$

Results & Discussion

For preparing calibration curve in HPLC 10 to 60 ppm caffeine standards were used to identify peak at retention time around 6.18-6.22 min. The peak area increases from the lowest standard to the highest. The slope of the curve was 56659, which was used for determination of caffeine in tea, coffee and soft drinks.

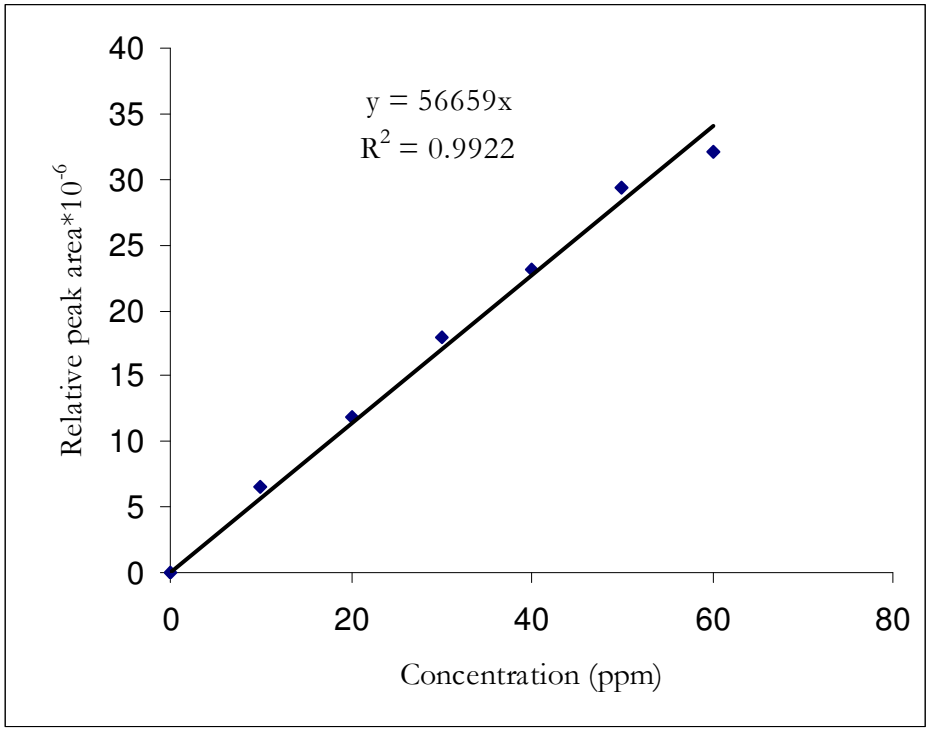


Figure 1: Calibration curve for caffeine in 50 mM KH_2PO_4 (pH=2), acetonitrile and methanol (40:8:2)

Table 1: Isolation and Purification of Caffeine from Tea and Coffee. (n=3)

Sample	Amount of Sample (g)	Amount of Caffeine after solvent extraction (g)	% of crude Caffeine before SPE	Amount of Caffeine after SPE Purification (g)	% of Caffeine after SPE	Standard Deviation (%)
Black Tea	5.0	0.352	7.04	0.167	3.34	0.88
Green Tea	5.0	0.244	4.88	0.122	2.44	0.79
Coffee	1.0	0.137	13.7	0.052	5.20	0.83

Table2: Percentage recovery of caffeine from black tea spiked with standard caffeine.

No. of observations	Amount of Black tea (g)	Amount of standard Caffeine added (g)	Amount of Caffeine obtained after SPE (g)	Spiked Result (%)
1	5	0.5	0.612	97
2	5		0.610	96
3	4		0.578	96
4	3		0.545	95

Table 3 : Characteristics of purified Caffeine isolated from Tea and Coffee.

Sample	Melting point (^{O}C)	λ_{max}	Retention Time (min)	R_f
Black Tea	235	270	6.19	0.63
Green Tea	236	270	6.20	0.63
Coffee	235	269	6.20	0.64

Table 4 : Characteristic IR-absorption bands of samples and standard.

Bonds	ν (cm^{-1}) (standard)	ν (cm^{-1}) (Black Tea)	ν (cm^{-1}) (Green Tea)	ν (cm^{-1}) (Coffee)
C=H	2860	2855	2853	2853
C-H	3100	3105	3105	3108
C=C	1645	1648	1652	1651
C=O	1690	1692	1696	1696
C-N	1240	1238	1237	1238
C=N	1590	1597	1595	1596

Table 5 : Caffeine determination in green tea, black tea and coffee. (by HPLC) (n=3)

Sample	Avg. RT (min)	Avg. Peak Area (a.u.)	Concentration of Caffeine (mg/L)	Standard Deviation (%)
Green Tea	6.20	559791	9.88	1.03
Black Tea	6.19	555258	9.80	0.98
Coffee	6.20	562723	9.93	0.97

Table 6 : Caffeine determination in various soft drinks (Cola-drinks) (by HPLC) (n=3)

Sample	Avg. RT (min)	Avg. Peak Area (a.u.)	Concentration of Caffeine (mg/L)	Standard Deviation (%)
Coca cola	6.22	739402	130.5	0.93
Double cola	6.22	920712	162.5	0.88
Uro cola	6.18	708240	125	0.89
RC cola	6.20	753567	133	0.97
Suncrest cola	6.20	617585	109	0.85
Pepsi cola	6.22	572258	101	0.87

Extraction of caffeine from tea and coffee was achieved by using chloroform as an extracting solvent. It was observed that the extraction efficiency of caffeine from various sources by using chloroform was much higher than other solvents. Table 1 shows extraction efficiency of crude caffeine from black tea, green tea and coffee. The amount of crude caffeine was found to be 7.04% from black tea, 4.88% from green tea and 13.7% from coffee (Table-1). We observed that black tea contained a higher percentage of crude caffeine than that of green tea and coffee contained a higher percentage of crude caffeine than that of both black and green tea. To purify the crude caffeine, SPE purification method was used. The amount of pure caffeine after SPE was found to be 3.34% from black tea, 2.24% from green tea and 5.20% from coffee (Table-1) After purification we have seen that coffee also contained a higher percentage of pure caffeine than that of both black and green tea. From the result of previous works it is evident that the SPE purification method resulted in a percentage of yields with greater purity.

5 g of black tea samples after solvent extraction and SPE purification was found to contain an average value of 0.167g of caffeine that corresponds to about 3.34% which is in good agreement with the literature value of 3.32 to 3.81[1,15]. However, the caffeine content of various sources varies with soil conditions and climate.

From the method of validation in case of only 0.5 g standard caffeine the amount of caffeine after solvent extraction and SPE was found as 0.45 g. It has been found also that the percent recovery of caffeine (black tea) spiked with standard caffeine was 95 to 97% (Table-2).

The pure white crystalline caffeine isolated from sources was found to melt at 235^0C. The average melting points of extracted crude caffeine of different samples were about 230^OC. It confirms the presence of impurities. Purification performed by SPE procedure gave the average melting point of about 235^OC. It can be concluded that the extracted product after purification was pure. This confirms that SPE purification was an essential step to obtain pure caffeine.

An UV-absorption spectrum of extracted purified caffeine was prepared by using UV-Visible spectrophotometer. The λ_{max} was found for various sources to be 271nm which is similar to that found in literature [16,17].

By the purified product (crystalline caffeine) a Thin Layer Chromatography was carried out on plates (Stationary phase: Silica gel coated on Aluminium) and the chromatographic process was developed using chloroform as mobile phase and visualized under UV-lamp. The R_f value was measured and it was found that commercially obtained caffeine showed similar R_f value. This confirms the purity of caffeine. The IR-spectrum of isolated caffeine showed similar absorption bands when compared with that of literature [7]. The IR-spectrum of Table 4 indicates the absolute purity of the purified caffeine.

We have developed a HPLC method for the determination of caffeine, which was carried by High Performance Liquid Chromatography (HPLC) instead of using UV-Visible spectrophotometer. We chose HPLC method for the determination of caffeine, because HPLC is the most widely used qualitative and quantitative determination and separation method. The method is popular because it is non-destructive and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique since it incorporates a wide choice of detection methods. With the use of post-column derivatization methods to improve selectivity and detection limits, HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as a separation method makes it a valuable separation tool in many scientific fields.

By using HPLC method, we determined the retention time and the relative peak area of extracted purified caffeine. The retention time of the purified caffeine and that of the standard caffeine were almost similar, which confirms the identity of caffeine. From relative peak area by using calibration curve the amount of extracted purified caffeine for different samples were also determined (Table 5).

Determination of the amount of caffeine was also carried out in several soft drinks that are available in Bangladesh. The commercial names of the analyzed soft drinks were Coca Cola, Double Cola, Uro Cola, RC cola, Suncrest Cola and Pepsi cola. The caffeine present in these soft drinks amounted to 130.5, 162.5,125,133,109 and 101 ppm respectively (Table 6). The caffeine contents of the soft drinks marketed in Bangladesh were found to be similar with that of the literature value [1].

Conclusion

A method has been developed for the extraction, purification of caffeine from tea, coffee and some soft drinks. Caffeine from the tea and coffee was extracted by liquid-liquid extraction and interferences were removed by employing solid-phase extraction (SPE).

The purified caffeine was then analyzed by HPLC. The soft drinks were directly analyzed by HPLC for their caffeine content. Solid phase extraction prior to HPLC was found to be an essential step for determining the amount of caffeine by HPLC. Characterization of caffeine was achieved by determining melting temperature, R_f value, IR

spectrum and UV spectrophotometry.

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