CAFFEINE IN VARIOUS SAMPLES AND THEIR ANALYSIS WITH HPLC – A REVIEW

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ABSTRACT
Coffee, Tea and soft drinks are very commonly used beverages in all over the world. The caffeine is the main stimulant occurred in all drinks. Caffeine stimulates the central nervous system, relaxation, myocardial stimulation, recreation etc. It can provide energy, decrease fatigue, enhance performance etc. Caffeine having medicinal properties so can be used along with other drugs for headache, stimulation, muscle relaxant etc. Up to certain limit caffeine is useful but overdose of caffeine starts side effects on the human body. There are various instrumental methods can be used for the determination of caffeine in plants, coffee, tea, soft drinks and pharmaceutical formulation in presence of other drugs. HPLC methods are the most common, reliable methods for the determination caffeine in complex sample. Very low concentration of caffeine can be determined with high accuracy and precision. Here in this review we have summarized various HPLC methods used for the caffeine analysis in various samples and complex mixtures with their chromatographic column, mobile phase, flow rate, detector etc.

Keywords: Caffeine, HPLC analysis, Beverages, coffee.

INTRODUCTION
Caffeine present in various beverages and foods. It is very important and essential to study about the caffeine. Various methods for the determination of caffeine are available. In present review we are studying various HPLC methods for the analysis of caffeine. The chemical formula is \((C_8H_{10}N_4O_2)\) and the common name for trimethylxanthine (systematic name is 1,3,7-trimethylxanthine or 3,7-dihydro-1, 3, 7-trimethyl-1Hpurine- 2, 6-dione). It is an addictive stimulant. It stimulates the central nervous system, heart rate, and respiration, has psychotropic (mood altering) properties, and acts as a mild diuretic.

Caffeine, theobromine and theophylline are natural alkaloids that are present in tea leaves, coffee, and cacao seeds and, therefore, in the food and beverages made from them. Caffeine is white crystalline powder with very bitter taste and occurs in many plants such as coffee beans, tealeaves and cocoa nuts etc. Caffeine is a stimulant commonly found in many foods, drinks. A chief ingredient of coffee is caffeine. Pure caffeine is 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 of 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.

The Caffeine is occurred in various plants, which 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. The amount of caffeine found in these products varies, the highest amounts are found in guarana containing 4-7%, tea leaves contains 3.5%, coffee beans having 1.1-2.2%, cola nuts 1.5% and cocoa beans is 0.03%. Caffeine is added to soft drinks as a flavoring agent, it is part of the overall profile of soft drinks. Consumers enjoy for soft drinks for refreshment, taste and hydration. Caffeine in cola drinks is added during the time of formulation Process.

Caffeine and theobromine have been found in more than 60 subtropical plant species. Many plants used for nonalcoholic beverages, such as tea (Camellia sinensis L.), coffee (Coffea arabica L.), cocoa (Theobroma cacao), and maté (Ilex paraguariensis), contain these purine alkaloids. Caffeine-containing products have been consumed for hundreds of years for their pleasant flavor and stimulating effects. Due to pharmacological properties of caffeine received increasing attention in food and pharmaceutical industries, which comprise stimulation of the central nervous system, peripheral vasoconstriction, relaxation of the smooth muscle and myocardial stimulation. The content of caffeine varies depending on tea type, which is directly attributed to their processing and leaf maturity. White tea, made from the youngest tea leaves contained the highest caffeine content, and mate and roasted mate teas the lowest.

USE OF CAFFEINE
Coffee is one of the most consumable beverages around the world today, often to provide a burst of energy when needed. On average, 90% of adults consume caffeine on a daily basis from beverages ranging from coffee, soda, tea,
and others. There are numerous benefits and drawbacks to caffeine consumption. Because caffeine is a stimulant, it can provide energy, decrease fatigue, and enhance motor performance\(^{18}\). Additionally, caffeine can help to maintain attention when needed. Coffee is often consumed by college students and other adults for this increase in energy and attention, so the amount of caffeine in various types of coffee is certainly of interest to the community. Caffeine is a naturally occurring substance found in the leaves, seeds or fruits of over 63 plants species worldwide\(^{6,8,11,34}\).

The levels of endogenous caffeine and theobromine were much higher in buds and young leaves of Coffea arabica L. than in fully developed leaves. Biosynthesis of caffeine from 14C-labeled adenine, guanine, xanthosine, and theobromine was observed, whereas other studies have indicated that there is no detectable incorporation of label into caffeine when theophylline and xanthine are used as substrates for in vivo feeds with leaves of C. Arabica\(^{15}\).

Caffeine is a contamination indicator of domestic water because it is anthropogenic origin and it is detected in both waste and surface water\(^{17}\). Surface water is contaminated due to waste water from septic disposal and pharmaceutical disposal areas\(^{18,19}\). Paxeus and Schroder (1996) reported that 37 µg /l of caffeine in to Swedish sewage treatment plant. The recommended daily dose of Caffeine for stimulation is 200 mg/day \(^{20}\). A dose of 10 g is lethal, which is equivalent to about 100 cups of coffee\(^{21,22}\). Recent epidemiological studies have seen an association between consumption of caffeine and risk of miscarriage\(^{23}\). High doses of caffeine are associated with various disorders affecting the central nervous system and cardiovascular system as well as increased gastric secretion and poor liver function\(^{24,25}\). This substance can induce addiction and anxiety\(^{26}\). Sporting organizations consider caffeine to be one of the prohibited nervous system stimulants, given that it increases the performance and diminishes fatigue. But concentration level of 12 µg/L in urine is permitted\(^{27-29}\) due to its part of normal daily diet. Concentration of Caffeine used in cola-based drinks is nearly 0.1 mg/mL, and manufacturer justify their use of this additive by claiming that caffeine enhances the aroma, although at such a concentration only a small percentage of consumers (approximately 8%) notice its presence\(^{30}\). Theobromine and theophylline are used for pharmaceutical purposes as bronchodilators and for vasodilators and also as mild muscle relaxants. They are used to prevent and treat shortness of breath caused by asthma and other respiratory disorders\(^{24}\).

Caffeine may be used in the treatment of acute circulatory failure. In either beverage or in nonprescription tablet form, it may be used to relieve fatigue since it increases the amount of urine flow. There are about 2000 non-prescription and about 1000 prescription drugs containing caffeine. Caffeine is administered in the treatment of mild respiratory depression caused by central nervous system depressants such as narcotic\(^{31}\). Caffeine stimulates the central nervous system, cardiac muscle, the respiratory system, and gastric secretion\(^{32}\).

Headache disorders are one of the most frequently reported symptoms and have been associated with impaired quality of life, increased incidence of depression, musculoskeletal pain, and disability\(^{33}\). Nearly 57 % males and 76 % females had one or more headache attack every month. Recent studies indicate that approximately 4 million men and 19 million women in the US population have migraine attacks\(^{34}\). 51% of adults indicated a headache attack in Europe in year \(^{34}\), and among German adolescents, the 3-months prevalence was 69%\(^{35}\). Pharmaceutical companies offer different kinds of analgesic and nonsteroidal anti-inflammatory drug mixtures, with or without ergot alkaloids and caffeine for acute headache therapy. Diverse combinations have been commercialized, mixing paracetamol (acetaminophen), aspirin (acetylsalicylic acid), or metamizole with caffeine and ergotamine\(^{36}\).

Caffeine is a pharmacologically active substance and depending on the dose, can be stimulate a mild central nervous system, it improve cardiac performance, increase brain circulation, and exhibit vasodilator and diuretic effect. It is also increase heartbeat rate, dilate blood vessels and elevate levels of free fatty acids and glucose in plasma. 1g of caffeine leads to insomnia, nervousness, nausea, ear ringing, flashing of light derillum and tremulousness. In cases of overdosing and in combination with alcohol, narcotics and some other drugs, these compounds produce a toxic effect, sometimes with lethal outcome\(^{37-40}\). Caffeine dose not accumulate in body over the course of time and is normally excreted within several hours of consumption\(^{4}\).

Caffeine is used both recreationally and medically to restore mental alertness when unusual weakness or drowsiness occurs. 100–200 mg dose of caffeine result in increased alertness and wakefulness, faster and clearer flow of thought, increased focus, and better general body coordination. It also results in restlessness, loss of fine motor control, headaches, and dizziness\(^{4}\). It is also noted that caffeine cannot replace sleep, and should be used only occasionally as an alertness aid. Caffeine is relatively safe for humans but it is substantially more toxic to other animals, such as dogs, horses, and parrots, because of their much poorer ability to metabolize the compound. Caffeine has a much greater effect on spiders than most other drugs\(^{41}\).

These alkaloids are contained in a variety of pharmaceutical products and drugs because they possess the properties such as it stimulate the central nervous system, it induce gastric secretions and it act as a diuretic\(^{37,38}\). Studies have also been done on these alkaloids to assess any antioxidant properties\(^{39}\). Recent epidemiological studies have seen an association between consumption of caffeine and risk of...
miscarriage. High doses of caffeine are associated with various disorders affecting the central nervous system and cardiovascular system as well as increased gastric secretion and poor liver function. This substance can induce addiction and anxiety.

**PHYSIOLOGICAL EFFECTS OF CAFFEINE**

The use of the mixture of acetaminophen and caffeine as an analgesic and antipyretic is well established in pharmaceutical formulation. Caffeine (CAF) in combination with acetylsalicylic acid (ASA) is used as an analgesic adjunct to enhance pain relief, although it has no analgesic activity of its own. Acute consumption of caffeine in combination with over-the-counter (OTC) analgesics such as ASA or acetaminophen increases their activity by as much as 40% depending on the specific type of pain involved. It is apparently due to the ability of caffeine to cause constriction of the cerebral blood vessels and possibly to facilitate the absorption of other drugs. The observed synergism of ASA and caffeine on the inhibition of PGE2 synthesis in microglial cells, a common model for the COX-2 inhibiting activity of non-steroidal anti-inflammatory drugs, may partly explain these effects. Caffeine alone might have analgesic properties for specific types of pain in humans and in human experimental pain models, but the overall evidence from clinical studies is weak. Caffeine crosses the placenta and enters the fetal circulation and its use at a pharmacological level has been associated with low birth weight. Excessive consumption during lactation may cause irritability and wakefulness in a breast-fed baby.

The effects of caffeine on human being depend on concentrations. Consuming high concentration of this compound causes various physiological and psychological effects such as relaxation of bronchial muscle, stimulation of the central nervous system, gastric acid secretion and diuresis. The increases in concentration of caffeine in vivo are also a key mark for various disorder including heart disease, kidney malfunction and asthma. Moreover, our sleeping habit, performance, and concentration are modified by caffeine. Caffeine has a tendency of rapidly and completely absorbed from gastrointestinal tract within a short period of time and distributed in the body; however, it is not removed from the circulation until metabolized initially into paraxanthine, theophylline and theobromine then into derivative of uric acid and diaminouracil, which is eventually removed from the circulation. So the plasma half life of caffeine in man, that is, the time required for its level to be diminished by 50% as a result of biotransformation and excretion is 5 to 6 h.

When the peak of plasma level of caffeine concentration is 15 to 30 M, effects like, mild anxiety, respiratory stimulation, cardiovascular effects, diuresis and increase in gastric secretion would be observed. When the levels are in between 150 to 200 M, a symptom of acute toxicity may appear. These include severe restlessness, excitement, muscular tension, twitching and cardiovascular disturbance such as tachycardia. The International Olympic Committee classified caffeine as a drug of abuse when it is present in human urine with concentration higher than 12 µg/ml.

About 200 mg of caffeine contains pharmacological effect. At this level, it stimulates the central nervous system, decreases fatigue leading to clearer flow of thoughts, sustained intellectual effort and a more perfect association of ideas with a better appreciation of sensory stimuli in man. At this level, it has a diuretic effect on the kidney hence affect fluid balance in the body. 1 g of caffeine leads to insomnia, nervousness, nausea, ear ringing, flashing of light derilium and tremulousness.

**ANALYSIS OF CAFFEINE**

Caffeine is very commonly occurred and used various soft drinks, hot drinks, beverages, medicines and available in various plant verities. Literature survey reviled that there are various methods has been reported since long back for the determination of caffeine present in various food stuffs and medicine. Now a day’s various sophisticated instruments are available for estimation of caffeine such as chromatographic techniques, chromatographic techniques coupled with mass spectrometer, UV spectrophotometer, Infra red spectrophotometry, capillary electrophoresis etc. Some methods are available for single component while some are used for combination with biological matrix and some with multicomponent in pharmaceutical formulations.

Some methods exist for the determination of caffeine, theobromine, and theophylline in different matrices such as food, drinks, and pharmaceutical products. The most widely used analytical techniques are mainly chromatography, such as high-performance liquid chromatography (HPLC) with spectrophotometric and amperometric detection, and capillary chromatography, such as gas chromatography, are also used as well as gas chromatography coupled with mass spectrometry prior to solid-phase extraction (SPE).

Several methodologies have been developed to determine these multi-component mixtures or to quantitative a single component. Among these, pharmaceuticals that contain metamizol, caffeine, or ergotamine, separately or in combination with other drugs, have been quantitatively determined with UV spectrophotometry, high-performance thin-layer chromatography (HPTLC) –UV, high-performance liquid chromatography (HPLC)-UV, capillary electrophoresis-UV, and flow injection analysis. However, no reference is available for the simultaneous determination of these three compounds by HPTLC or HPLC extraction via a special surface sampling probe followed by electrospray ionization (ESI). Caffeine has been also determined in combination with other drugs using UVspectrophotometer, High-Performance Liquid Chromatography (HPLC), Gas chromatography, and Mass spectrometry in pharmaceutical preparations.
Table 1: HPLC Methods used for analysis of Caffeine with various Chromatographic conditions

<table>
<thead>
<tr>
<th>Caffeine sample</th>
<th>Method</th>
<th>Mobile phase</th>
<th>Detection</th>
<th>Flow rate</th>
<th>Ref.</th>
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<tr>
<td><strong>Pharmaceutical Dosage form</strong></td>
<td>RP-HPLC</td>
<td>Water and methanol (60:40)</td>
<td>C18 column (4.5 mm x 250 mm; 5 µm particle size)</td>
<td>272 nm</td>
<td>1 ml/min</td>
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<tr>
<td></td>
<td>RP-HPLC</td>
<td>Methanol and water eluent (40:60)</td>
<td>LiChroCART 250-4 Puroluer RP.18 column (4.6 x 250 mm, particle size 5 µm)</td>
<td>249 and 273 nm</td>
<td>0.5 ml/min-1</td>
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<tr>
<td><strong>Tablets form</strong></td>
<td>RP-HPLC</td>
<td>90:10 (v/v) aqueous ortho- phosphoric acid (pH 2.1) – acetone</td>
<td>250 mm x 4.6 mm, 5-µm particle diam., LiChropur 60 – C18, B</td>
<td>205 nm diol diode detector, reversed phase C18 column</td>
<td>1.5 ml / min</td>
</tr>
<tr>
<td></td>
<td>RP-HPLC</td>
<td>Isocratic elution with methanol and 1% (v/v) acetic acid (1:4)</td>
<td>Merck RP select B 250-46.4 mm LD. reversed phase C18 column</td>
<td>JV 270 nm</td>
<td>1 ml / min</td>
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<td><strong>Caffeine (CA) in traditional Chinese medicine</strong></td>
<td>RP-HPLC</td>
<td>Methanol –water–acetic acid (80:19:1) (v/v) –isocratic</td>
<td>Supercosil LC-18 col (250 mm x 4.6 mm, 5 µm, Supelco, Sigma-Aldrich, and a Supelguard LC 18 precolumn (20 mm x 2.1 mm))</td>
<td>JV 275 nm</td>
<td>1 ml / min</td>
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<td><strong>Caffeine and theobromine in coffee brews</strong></td>
<td>RP-HPLC</td>
<td>Methanol with 20 µm ammonium acetate buffer (pH 7.5) (20:80:5) VV</td>
<td>Phenomenex Kinetex 2.6 um XB-C18</td>
<td>JV 272 nm</td>
<td>1 ml / min</td>
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<td><strong>Caffeine, theobromine, and gallic acid from tea</strong></td>
<td>RP-HPLC</td>
<td>Water – acetanilide (90:10)</td>
<td>Kinetex 2.6u XB-C18 100A column</td>
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<td><strong>Caffeine, aspartame, benzoic acid, saccharin in sugar-free beverage</strong></td>
<td>RP-HPLC</td>
<td>methanol and an aqueous solution phosphate buffer pH 3</td>
<td>Phenomenex Kinetex 2.6u XB-C18 100A.; 50 x 4.6 mm.</td>
<td>JV 220 &amp; 2.70 nm</td>
<td>1 ml/min</td>
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<td>RP-HPLC</td>
<td>Gradient elution method of a 90:10 (v/v) phosphate buffer/methanol</td>
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<td>JV 272nm</td>
<td>1 ml/min</td>
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<td>0.5 ml/min</td>
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<td><strong>Caffeine Content in Tea and Male Tea</strong></td>
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<tr>
<td><strong>Caffeine and Acetaminophen</strong></td>
<td>HPLC</td>
<td>Phosphate buffer (pH 5.5); methanol (60:40)</td>
<td>column (C18, 250 mm X 4.6 mm, 5 µm, Shim-pack, Japan)</td>
<td>JV 273 nm</td>
<td>1 ml/min</td>
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<td><strong>Tea catechins and theaflavins</strong></td>
<td>HPLC gradient elution</td>
<td>(A) 5% (v/v) acetanilide with 0.035% (v/v) trifluoroacetic acid (B) 50% (v/v) acetanilide with 0.025% (v/v) TFA</td>
<td>Partispherse 5 C18, 5 µm, 110 mm34.6 mm1.6 ID</td>
<td>JV 205 nm</td>
<td>1 ml/min</td>
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<tr>
<td><strong>Caffeine in tea leaves</strong></td>
<td>RP HPLC</td>
<td>(methylene-water–acetic acid, 40:59:1, v/v)</td>
<td>75 mm 3 mm I.D., 3 mm, ODS-UG-3, Nomural Chemical</td>
<td>JV 272 nm</td>
<td>0.6 ml/min</td>
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<td>0.1% HCl/ACN : 98:2 (v/v)</td>
<td>Kovalis MS-C18 (1.5 µm, 33mm x 4.4mm i.d., and Nucleosil 100-5 C18 (5 µm, 100 Å), 250mm4.4mm)</td>
<td>JV 280 nm</td>
<td>1 ml/min</td>
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<td>1 ml/min</td>
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<td>Shim-pack VP-ODS with internal diameter 4.6 mm and length 250mm</td>
<td>270 nm.</td>
<td>1.3 ml/min</td>
</tr>
<tr>
<td><strong>Paracetamol, Caffeine and Dipyrone</strong></td>
<td>HPLC</td>
<td>0.01 M KH2PO4—methylanol-acetanilide-isopropyl alcohol (420: 20: 30: 5) (v/v/v/v)</td>
<td>-Bondapak C8 column (5 µm, 250 mm X 4.6 mm 6.4 µm ID);</td>
<td>215 nm</td>
<td>1.0 ml/min</td>
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<td><strong>paracetamol, pseudoprophene, caffeine and chlorophenamine maleate in dosage form</strong></td>
<td>RP-HPLC</td>
<td>Gradient elution - A-phosphate buffer (1.0g of KH2PO4 in 4 to 1000ml of HPLC water and mixed) and sol-B: acetanilide</td>
<td>C18 (150mm, 4.6mm and 3µm) column</td>
<td>210nm</td>
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<td>Gradient elution – A -10 mM citric acid and B- methanol. A; B ratio of 85:15 from 0 to 5 minutes and will increase to 60:40 at 40 to 85 minutes</td>
<td>150x 4.6 mm i.d. Merck Superspher 100 RP (Reversed Phase) 18 column (5 µm particle size)</td>
<td>276 nm</td>
<td>Nor Hanishah MY,</td>
</tr>
<tr>
<td><strong>Catechins, Theaflavins, Caffeine, and Theobromine in 77 Teas</strong></td>
<td>RP-HPLC</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Caffeinated Energy Drinks</strong></td>
<td>HPLC</td>
<td>Gradient elution – A) Amm. Acetate: B) Water, C) Acetanilide A:B:C – 9:1:90</td>
<td>Ascentis express HILIC, 10 cm x 3mm ID, 2.7 µm particle size.</td>
<td>JV 254 nm</td>
<td>0.6 ml/min</td>
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<tr>
<td><strong>Caffeinated Energy Drinks</strong></td>
<td>HPLC</td>
<td>Gradient elution – A) 0.1 % TFA (v/v) in water B) 0.1 % TFA (v/v) acetanilide</td>
<td>Ascentis express HILIC, 10 cm x 3mm ID, 2.7 µm particle size.</td>
<td>JV 254 nm</td>
<td>0.6 ml/min</td>
</tr>
<tr>
<td><strong>Polyphenolic Separation Of Teas</strong></td>
<td>HPLC</td>
<td>Gradient Elution- A) (100% H2O) and B) (60% Methanol and 40% H2O) each to pH 2.4</td>
<td>Dionex 250 x 4.6 mm Acclaim 120-C18 column</td>
<td>JV 280nm.</td>
<td>0.8 ml/min</td>
</tr>
<tr>
<td><strong>Caffeine content of tea and instant coffee brands</strong></td>
<td>HPLC</td>
<td>water, acetic acid, methanol (79.9, 0.1 and 20) v/v.</td>
<td>Reversed phase ODS, 250 x 4.6 mm</td>
<td>JV 278 nm</td>
<td>1 ml/min</td>
</tr>
<tr>
<td><strong>Caffeine paracetamol, pseudoprophene, and chlorophenamine maleate</strong></td>
<td>HPLC</td>
<td>Gradient elution A: phosphate buffer (KH2PO4 1g/1000) and sol-B: acetanilide.</td>
<td>C18 (150mm, 4.6mm and 3µm) column</td>
<td>JV 210 nm</td>
<td>1 ml/min</td>
</tr>
<tr>
<td><strong>Caffeine in Common Sweeteners and Additives</strong></td>
<td>UHPLC</td>
<td>A: 0.1% TFA in water B: 0.1% TFA in acetanilide</td>
<td>Restek® Pinephyl DB C18, 3 µm, 100 x 2.1 mm, PerkinElmer Brownlee® Analytical C-18, 250 x 4.6 mm</td>
<td>Hexar FX PDA UHPLC 214nm</td>
<td>0.7 ml/min. from 1.0 ml/min</td>
</tr>
</tbody>
</table>
Several HPLC methods have been reported for the determination of marker constituents in Chinese medicinal prescriptions. Although many HPLC methods have been developed for the determination of caffeine in tea or preparations, a number of SPE methods have been developed to measure CA in biological samples. There are various HPLC methods have been reported in various samples containing caffeine.

Analysis methods are also described in the pharmacopeia. Several methods have been reported for the determination of caffeine in food or beverages. Few methods have been reported for the simultaneous determination of acetaminophen and caffeine in tablet dosage form.

Several methods have been reported for the determination of caffeine in food or beverages. Simultaneous determination of acetaminophen with other drugs has also been reported. But only few methods have been reported for the simultaneous determination of acetaminophen and caffeine in tablet dosage form.

Other methods

Another most widely used methods for the determination of caffeine in beverages are UV-Vis spectrophotometer and partial least square. UV-Vis spectrophotometry, luminescence, derivative spectrophotometry, Fourier transform infrared spectroscopy, near infrared reflectance (NIR Reflectance) spectrometry, and capillary electrophoresis are very commonly used techniques. Spectrophotometer method is fast, simple, accurate, reproducible and inexpensive procedure as compared to other methods; however, it is not possible to determine caffeine directly in coffee beans by conventional UV-Vis absorption measurement due to the spectral overlap of UV absorbing substances in the sample. The derivative spectrophotometry is relatively easy; but, it is not reliable for the small concentration of caffeine in samples. By HPLC methods, many caffeine contents were determined in various foods using different procedures since it provides the most reliable method. However, the use of expensive equipments and the demand for more operator attention prevents its applications in small industrial laboratories where only a few analyses are performed each day.

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