

Study of Microbial Degradation of Caffeine from Different Samples of Coffee

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Coffee is the most commonly consumed product worldwide. The caffeine present in coffee acts as a central nervous system stimulant. Apart from coffee, caffeine is also found in tea, yerba mate and guarana berries. It is a legal psychoactive substance available all over the world. Due to the harmful effects of caffeine on the body, the present studying aims at studying the degradation of caffeine by microorganisms so that the ill effects of the compound can be overcome. Caffeine was extracted from different types of coffee and its degradation by *Bacillus subtilis* and *Pseudomonas aeruginosa* was studied. The concentration to be used was detected by MIC and degradative activity was obtained by HPLC.

Key words: Caffeine, *Bacillus subtilis*, *Pseudomonas aeruginosa*, MIC, HPLC.

Caffeine (also spelled *caffein*)¹ is a bitter, white crystalline xanthine alkaloid that is a psychoactive stimulant drug. Caffeine is found in varying quantities in the beans, leaves, and fruit of some plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding on the plants². It is most commonly consumed by humans in infusions extracted from the bean of the coffee plant and the leaves of the tea bush, as well as from various foods and drinks containing products derived from the kola nut. Other sources include yerba mate, guarana berries, and the Yaupon Holly.

In humans, caffeine acts as a central nervous system (CNS) stimulant, temporarily warding off drowsiness and restoring alertness. Caffeine is the world's most widely consumed psychoactive substance³, but, unlike many other psychoactive substances, is legal and unregulated in nearly all jurisdictions. Beverages containing caffeine, such as coffee, tea, soft drinks, and energy drinks, enjoy great popularity. The U.S. Food and Drug Administration (FDA) lists caffeine as a "multiple purpose generally recognized as safe food substance"⁴.

Caffeine from coffee or other beverages is absorbed by the stomach and small intestine within 45 minutes of ingestion and then distributed throughout all tissues of the body⁵. It is eliminated by first-order kinetics⁶. Caffeine can also be ingested rectally, evidenced by the formulation of suppositories of ergotamine tartrate and caffeine (for the relief of migraine)⁷ and chlorobutanol and caffeine (for the treatment of hyperemesis)⁸.

The biological half-life of caffeine — the time required for the body to eliminate one-half of the total amount of caffeine — varies widely among

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individuals according to such factors as age, liver function, pregnancy, some concurrent medications, and the level of enzymes in the liver needed for caffeine metabolism. In healthy adults, caffeine's half-life is approximately 4.9 hours⁹. In women taking oral contraceptives, this is increased to 5–10 hours,¹⁰ and in pregnant women the half-life is roughly 9–11 hours.¹¹

Caffeine can accumulate in individuals with severe liver disease, increasing its half-life up to 96 hours.¹² In infants and young children, the half-life may be longer than in adults; half-life in a newborn baby may be as long as 30 hours. Other factors such as smoking can shorten caffeine's half-life.¹³ Fluvoxamine (Luvox) reduced the clearance of caffeine by 91.3%, and prolonged its elimination half-life by 11.4-fold; from 4.9 hours to 56 hours.⁹

Caffeine is metabolized in the liver by the cytochrome P450 oxidase enzyme system (to be specific, the 1A2 isozyme) into three metabolic dimethylxanthines,¹⁴ each of which has its own effects on the body:

- Paraxanthine (84%): Has the effect of increasing lipolysis, leading to elevated glycerol and free fatty acid levels in the blood plasma.
- Theobromine (12%): Dilates blood vessels and increases urine volume. Theobromine is also the principal alkaloid in the cocoa bean, and therefore chocolate.
- Theophylline (4%): Relaxes smooth muscles of the bronchi, and is used to treat asthma. The therapeutic dose of theophylline, however, is many times greater than the levels attained from caffeine metabolism.

Each of these metabolites is further metabolized and then excreted in the urine.

MATERIALS AND METHODS

The caffeine was extracted from three different coffee samples which included Nescafe, Bru and a local brand. The test organisms used were *Bacillus subtilis* (Gram positive cocci) and *Pseudomonas aeruginosa* (Gram negative bacilli) to study the degradation of the extracted caffeine.

Extraction of caffeine from coffee samples

1 gm of coffee sample was dissolved in 250 ml of distilled water by boiling for 5 minutes.

This solution was then filtered through buchner filter. 25 ml of 10% lead acetate was added to the filtrate and mixed by boiling for 5 minutes. This solution was mixed with 30–50 ml of chloroform. After mixing allow it to stand. Two layers are formed in which lower layer being chloroform, is separated out. Anhydrous sodium sulphate is added to the remaining solution for the removal of water and any dissolved salts. This is placed in water bath at 70–90°C for evaporation. This was then kept at 37°C for 24 hours. White crude caffeine was obtained at the bottom of the flask.

Minimum inhibitory concentration (MIC) of caffeine

The MIC of caffeine was found using a range of 1% to 20% of caffeine since 20% of crude extract of caffeine was obtained from the samples under study. MIC gives the minimum concentration inhibitory to the organisms so as to use the tolerable concentration for further studies. The culture suspensions used had an optical density of 0.1 at 540nm. The stock was 20% caffeine and sterile nutrient agar without sugar was used as diluents.

Growth curve of the test organisms in presence of caffeine

The MIC results showed that 6% of caffeine was tolerable to the test organisms. Hence the growth curve was studied using 6% caffeine in the growth medium. The growth curve was essential to find the time required for reaching the stationary phase. The initial optical density of the cultures was 0.1 at 540nm 100 ml volume. The readings were taken until the organisms reached the stationary phase. 2ml of aliquots were taken out after every hour to find the growth and the same was used for estimating caffeine utilization. Graph a, b and c.

Estimation of caffeine degradation

The aliquots obtained from the above procedure, were estimated for the concentration of the caffeine by using isocratic HPLC. The mobile phase used was – Methanol: water : acetic acid in the ratio of 40:60:1.6.

Chromatogram 1 to 12 shows the degradative activity

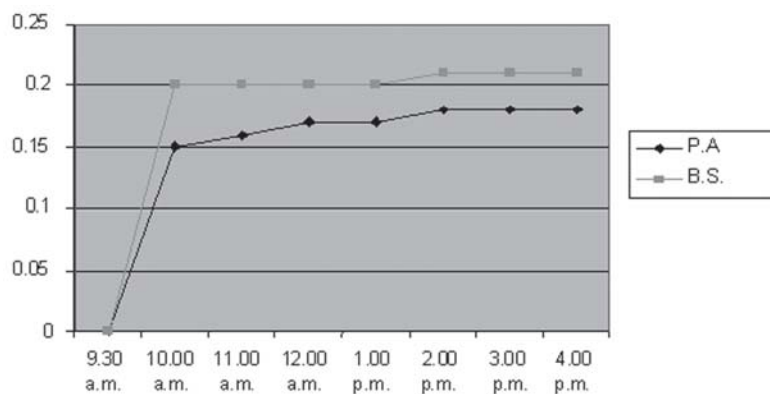
RESULTS

Minimum inhibitory concentration of Caffeine

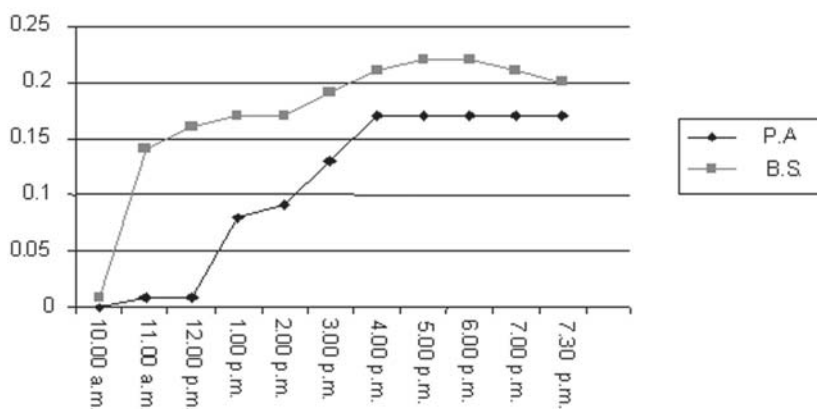
The MIC was found to be in the range of

6% to 7% for both the microorganisms. Hence a concentration less than 6% was used for the growth curve study and degradative analysis.

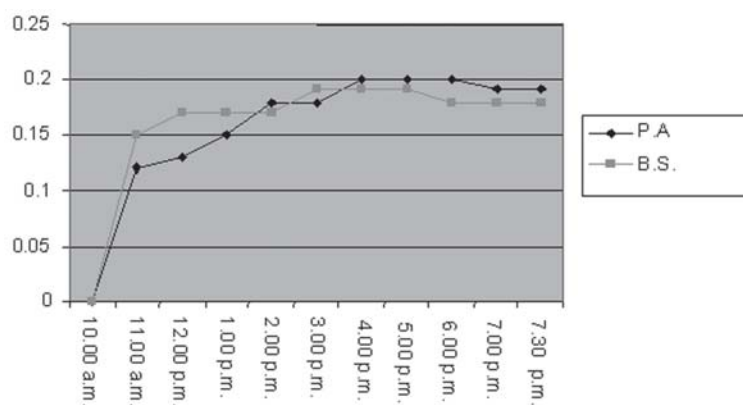
Growth curves



a) Growth curve of both organisms when grown in presence of caffeine from Nescafe



b) Growth curve of both organisms when grown in presence of caffeine from Bru.



c) Growth curve of both organisms when grown in presence of caffeine from Loose coffee sample

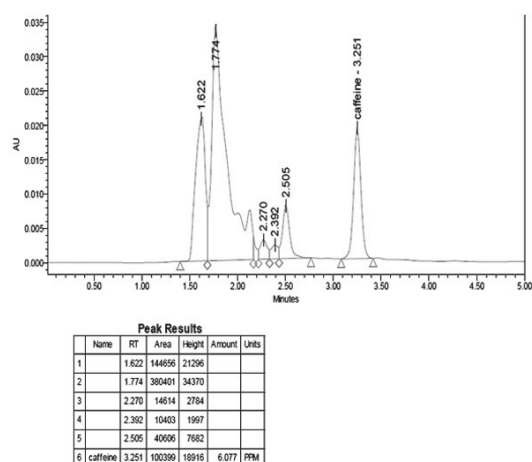
Legends:

P.A – *Pseudomonas aeruginosa*

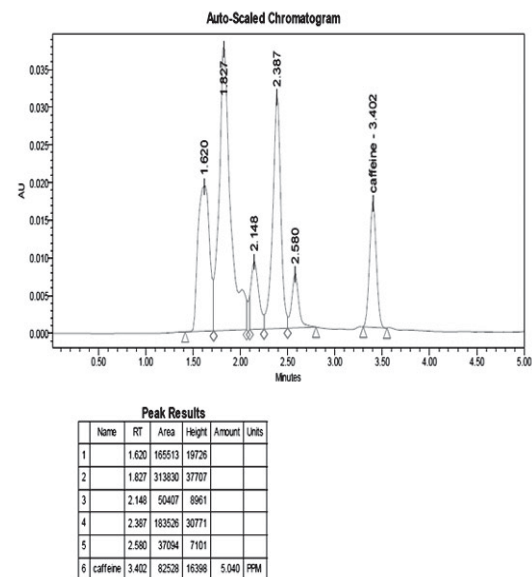
B.S – *Bacillus subtilis*

Estimation of caffeine degradation by HPLC

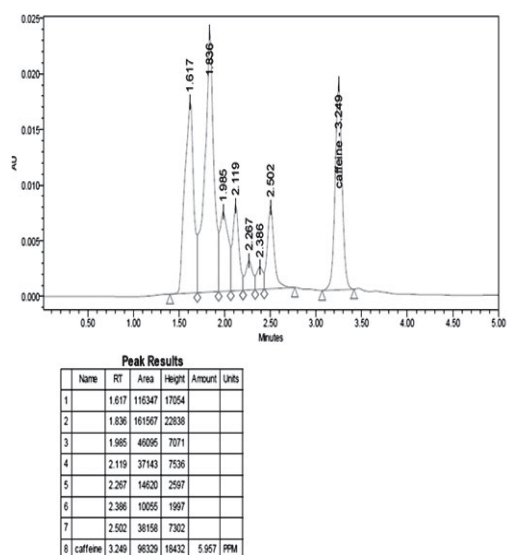
Chromatogram 1: Sample - Bru , Organism - *Bacillus subtilis* (0 hour)



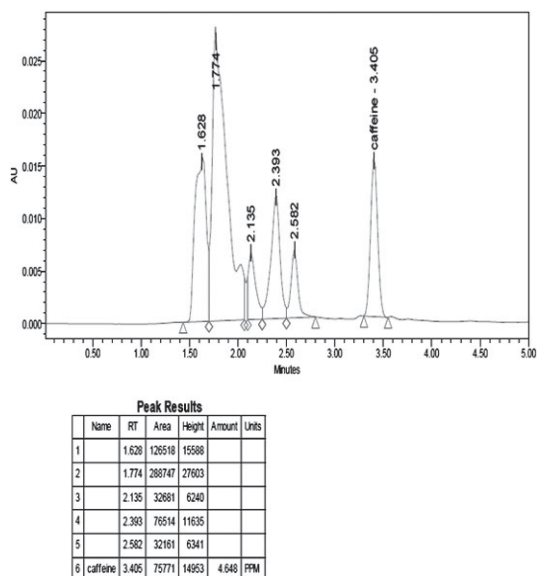
Chromatogram 2 : Sample - Bru , Organism - *Bacillus subtilis* (5 hours)

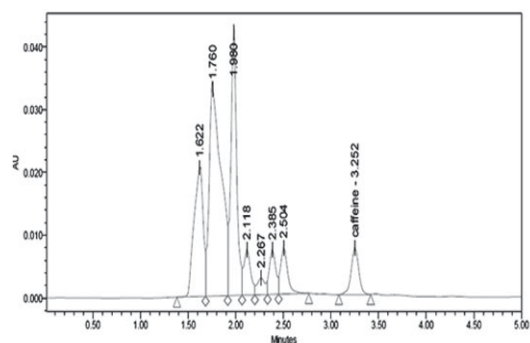


Chromatogram 3: Sample - Bru , Organism - *Pseudomonas aeruginosa* (0 hour)

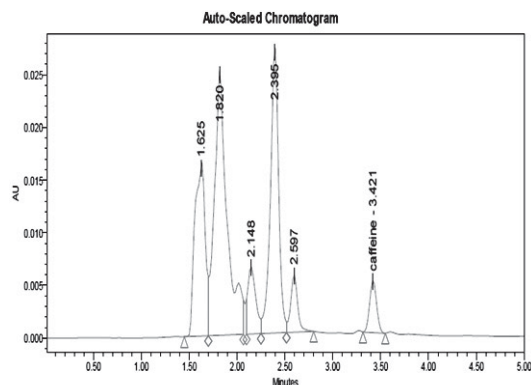


Chromatogram 4 : Sample - Bru , Organism - *Pseudomonas aeruginosa* (5 hours)

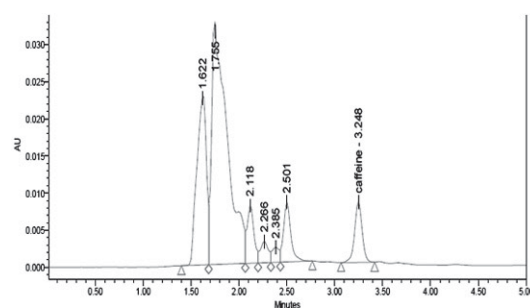


Chromatogram 5 : Sample - Nesscafe , Organism - *Bacillus subtilis* (0 hour)

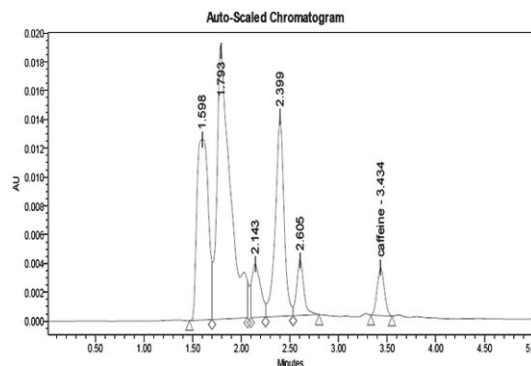
Name	RT	Area	Height	Amount	Units
1	1.622	136420	20729		
2	1.760	292529	34248		
3	1.980	161788	41199		
4	2.118	36454	7112		
5	2.267	16484	2649		
6	2.385	33090	7023		
7	2.504	38018	7320		
8	caffeine	3.252	41217	7480	2.642 PPM

Chromatogram 6 : Sample - Nesscafe , Organism - *Bacillus subtilis* (5 hours)

Name	RT	Area	Height	Amount	Units
1	1.625	123464	16412		
2	1.820	232366	24552		
3	2.148	36300	6311		
4	2.395	161413	26836		
5	2.597	27619	5364		
6	caffeine	3.421	34915	4902	1.696 PPM

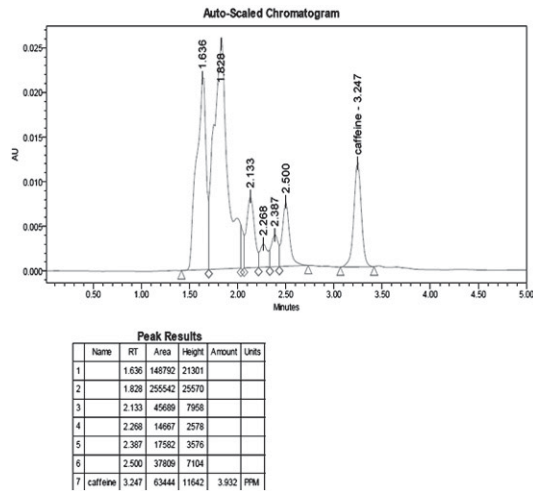
Chromatogram 7 : Sample - Nescafe , Organism - *Pseudomonas aeruginosa* (0 hour)

Name	RT	Area	Height	Amount	Units
1	1.622	155892	23013		
2	1.755	368196	32452		
3	2.118	38800	7631		
4	2.266	16688	2845		
5	2.385	10623	2002		
6	2.501	42353	7944		
7	caffeine	3.248	44441	6122	2.830 PPM

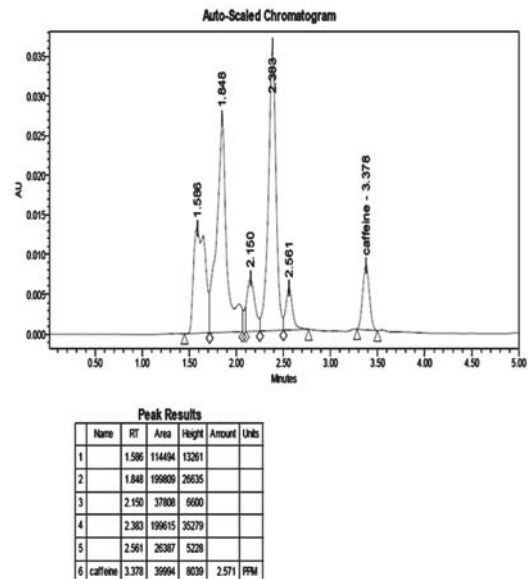
Chromatogram 8 : Sample - Nescafe, Organism - *Pseudomonas aeruginosa* (5 hours)

Name	RT	Area	Height	Amount	Units
1	1.598	101961	12561		
2	1.793	180007	19114		
3	2.143	22676	3688		
4	2.399	86003	13758		
5	2.605	19432	3810		
6	caffeine	3.434	16979	3324	1.236 PPM

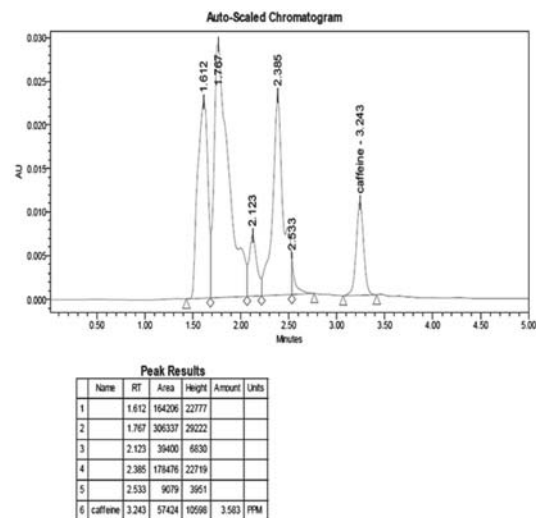
Chromatogram 9 : Sample - Loose coffee , Organism - *Bacillus subtilis* (0 hour)



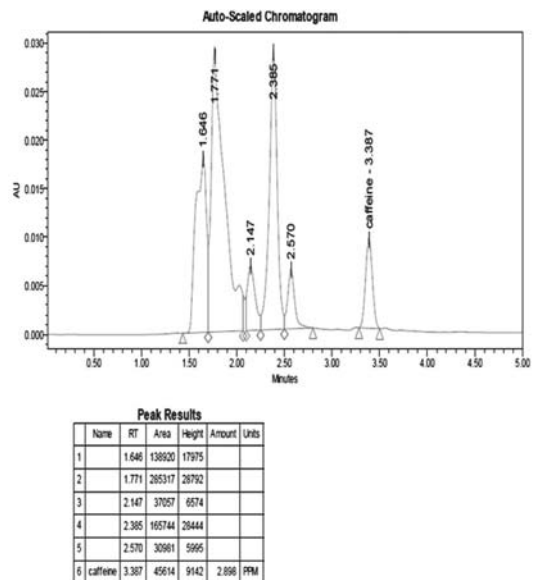
Chromatogram 10 : Sample - Loose coffee , Organism - *Bacillus subtilis* (5 hours)



Chromatogram 11 : Sample - Loose coffee , Organism - *Pseudomonas aeruginosa* (0 hour)



Chromatogram 12 : Sample - Loose coffee , Organism - *Pseudomonas aeruginosa* (5 hours)



The test organisms achieved the stationary phase after 4-5 hours when grown in a medium containing the caffeine extracts. The concentration of caffeine used for growth curve study was based on the results of MIC which was 6% of caffeine. The growth curve is shown in Graph a, b and c. Considering the growth curve obtained, the degradation of the caffeine from different coffee samples was studied and analysed by HPLC. The HPLC results are shown in Chromatograms 1 to 12.

The maximum degradation of caffeine obtained from Nescafe was shown by *Pseudomonas aeruginosa* i.e. around 57% whereas least degradation of caffeine from loose coffee sample i.e. 19%.

Bacillus subtilis showed almost similar degradation of caffeine from Nescafe as well as loose coffee i.e. 36% and 35% respectively, but reduced degradation of caffeine from Bru.

DISCUSSION

Coffee is processed by the dry method giving an expensive amount of husk, which in turn is used as an organic fertiliser. The presence of tannins and caffeine diminish the acceptability and palatability of husks by animals. Coffee pulp, the waste generated in large quantities during wet method of coffee cherry processing contains 8.25% protein and 23-27% fermentable sugars on dry weight basis. In spite of such high nutrient content, the coffee pulp cannot be an animal feed, mainly due to its toxic components such as caffeine, tannins, phenols and other polyphenols. Consequently most of the coffee pulp remains unutilised and a need exists for its treatment by appropriate biological waste treatment process to overcome severe environmental pollution problem.

Being cost intensive in nature, the coffee pulp forms a part of the waste adversely affect the cost of production of coffee and hence, it is generally avoided by the coffee processors. The coffee pulp forms a major source of the pollution of rivers, lakes and environment in the vicinity of the coffee processing sites. Elimination of the toxic and anti-physiological factors in the coffee pulp can lead to much cheaper and abundant alternative source of nutrient for use as animal feed. Caffeine degrading enzymes such as caffeine demethylase, theobromine demethylase, heteroxanthine

demethylase, caffeine oxidase and xanthine oxidase that are produced by several caffeine degrading bacterial species can be used to degrade caffeine.

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REFERENCES

1. "Caffeine or caffein". *Collins English Dictionary* (10th ed.). HarperCollins Publishers. 2009. <http://dictionary.reference.com/browse/caffeine>. Retrieved 2010-11-08.
2. Nathanson, J. "Caffeine and related methylxanthines: possible naturally occurring pesticides". *Science*. 1984; **226**(4671): 184-7. doi:10.1126/science.6207592. PMID 6207592.
3. Lovett, Richard (24 September 2005). "Coffee: The demon drink?". *New Scientist* (2518). <http://www.newscientist.com/article.ns?id=mg18725181.700>. Retrieved 2009-08-03.
4. 21CFR 182.1180". *U.S. Code of Federal Regulations*. U.S. Office of the Federal Register. 2003-04-01. p. 462. http://edocket.access.gpo.gov/cfr_2003/aprqt/21cfr182.1180.htm. Retrieved 2009-08-03.
5. Liguori, A. "Absorption and Subjective Effects of Caffeine from Coffee, Cola and Capsules". *Pharmacology Biochemistry and Behavior*. 1997; **58**: 721-6. doi:10.1016/S0091-3057(97):00003-8.
6. Newton, R.; Broughton, L. J.; Lind, M. J.; Morrison, P. J.; Rogers, H. J.; Bradbrook, I. D. "Plasma and salivary pharmacokinetics of caffeine in man". *European Journal of Clinical Pharmacology*. 1981; **21** (1): 45-52. doi:10.1007/BF00609587. PMID 7333346.
7. Graham, John R. "Rectal Use of Ergotamine Tartrate and Caffeine Alkaloid for the Relief of Migraine". *New England Journal of Medicine*. 1954; **250**(22): 936-8. doi:10.1056/NEJM195406032502203. PMID 13165929.
8. Brødback HB, Damkier P. "[The treatment of hyperemesis gravidarum with chlorobutanol-caffeine rectal suppositories in Denmark: practice and evidence]" (in Danish). *Ugeskr. Laeg*. 2007; **169** (22): 2122-3. PMID 17553397.
9. Drug Interaction: Caffeine Oral and Fluvoxamine Oral Medscape Multi-Drug Interaction Checker

- 2010.
10. Meyer, FP; Canzler, E; Giers, H; Walther, H (1991). "Time course of inhibition of caffeine elimination in response to the oral depot contraceptive agent Deposiston. Hormonal contraceptives and caffeine elimination". *Zentralbl Gynakol* **113** (6): 297–302. PMID 2058339.
11. Ortweiler, W; Simon, HU; Splinter, FK; Peiker, G; Siegert, C; Traeger, A. "Determination of caffeine and metamizole elimination in pregnancy and after delivery as an in vivo method for characterization of various cytochrome p-450 dependent biotransformation reactions". *Biomed Biochim Acta*. 1985; **44** (7–8): 1189–99. PMID 4084271.
12. Bolton, Ph.D., Sanford. "Caffeine: Psychological Effects, Use and Abuse". *Orthomolecular Psychiatry*. 1981; **10**(3): 202–11.
13. Springhouse (January 1, 2005). *Physician's Drug Handbook; 11th edition*. Lippincott Williams & Wilkins. ISBN 1-58255-396-3.
14. "Caffeine". The Pharmacogenetics and Pharmacogenomics Knowledge Base. <http://www.pharmgkb.org/do/serve?objId=PA448710&objCls=Drug &tabType=Properties #biotransformation>. Retrieved 2010-10-25.