Brazilian Journal of health Review

Analytical validation of caffeine quantification method in decaffeinated coffee by HPLC-UV

Validação analítica do método de quantificação de cafeína em café descafeinado por HPLC-UV

Maria Fernanda Ferreira de Lima
Bacharel em Farmácia pelo Centro Universitário Tabosa de Almeida
Instituição: Centro Universitário Tabosa de Almeida
Endereço: Avenida Portugal, 584 - Bairro Universitário, Caruaru - PE, Brasil
E-mail: nandadelyma@gmail.com

Gislainy Isrraiany da Silva França
Bacharel em Farmácia pelo Centro Universitário Tabosa de Almeida
Instituição: Centro Universitário Tabosa de Almeida
Endereço: Avenida Portugal, 584 - Bairro Universitário, Caruaru - PE, Brasil
E-mail: gislainyfrancaa@gmail.com

Diogo Jorge Fragoso de Souza
Bacharel em Farmácia pela Universidade Federal de Pernambuco
Instituição: IMEC - Indústria de Medicamentos Custódia
Endereço: Avenida Gerson Gonçalves de Lima, 1608 - Bairro Macambira, Custódia - PE, Brasil
E-mail: dfragoso563@gmail.com

Analúcia Guedes Silveira Cabral
Doutora em Produtos Naturais e Sintéticos Bioativos pela Universidade Federal da Paraíba
Instituição: Centro Universitário Tabosa de Almeida
Endereço: Avenida Portugal, 584 - Bairro Universitário, Caruaru - PE, Brasil
E-mail: analuciaguedes@asces.edu.br

Clayton Anderson de Azevedo Filho
Doutor em Ciências Farmacêuticas pela Universidade Federal de Pernambuco
Instituição: Centro Universitário Tabosa de Almeida
Endereço: Avenida Portugal, 584 - Bairro Universitário, Caruaru - PE, Brasil
E-mail: claytonfilho@asces.edu.br

Brazilian Journal of health Review

Ellison Neves de Lima
Doutor em Ciências Farmacêuticas pela Universidade Federal de Pernambuco
Instituição: Centro Universitário Tabosa de Almeida
Endereço: Avenida Portugal, 584 - Bairro Universitário, Caruaru - PE, Brasil
E-mail: ellisonlima@asces.edu.br

Carlos Eduardo Miranda de Sousa*
Doutor em Ciências Farmacêuticas pela Universidade Federal de Pernambuco
Instituição: Centro Universitário Tabosa de Almeida
Endereço: Avenida Portugal, 584 - Bairro Universitário, Caruaru - PE, Brasil
E-mail: carlossousa@asces.edu.br

ABSTRACT

This study aimed to determine the caffeine content and to verify that the contents of decaffeinated coffee are within the standards determined by regulatory agencies. This paper describes a rapid (7.0 min) and sensitive (LLOQ 0.8 µg/mL) analytical method for the quantitation of Caffeine (CFF) in decaffeinated coffee. The method is based on High-performance Liquid chromatography-Ultraviolet (HPLC-UV) with a wavelength of 285nm. Sample preparation involved liquid-liquid extraction with chloroform. The chromatographic separation was achieved on a ACE C18 (150 x 4,6 mm) reversed-phase column and a mobile phase containing acetonitrile/water (20:80 v/v), in isocratic conditions. The assay was linear in the concentration range of 10 – 100 µg/mL. The mean recovery for CFF was 99.89%. Intra- and inter-day precision (R.S.D.) were < 1.5 % and <1.82 %, respectively and the accuracy (R.E.) was in the range ± 2.14 %.The results, verified by the validation, showed an adequate technique to be applied in the samples evaluated, in addition to a simple, fast execution methodology, with lower costs.

Keywords: Caffeine, Decaffeinated coffee, HPLC-UV, Validation

RESUMO

Este estudo teve como objetivo determinar o teor de cafeína e verificar se o conteúdo do café descafeinado está dentro dos padrões determinados pelas agências reguladoras. Este artigo descreve um método analítico rápido (7,0 min) e sensível (LLOQ 0,8 µg / mL) para quantificação de cafeína (CFF) em café descafeinado. O método é baseado em cromatografia líquida de alta eficiência - ultravioleta (CLAE-UV) com comprimento de onda de 285nm. A preparação das amostras envolveu extração líquida com clorofórmio. A separação cromatográfica foi realizada em uma coluna de fase reversa ACE C18 (150 x 4,6 mm) e uma fase móvel contendo acetonitrila / água (20:80 v / v), em condições isocráticas. O ensaio foi linear na faixa de concentração de 10 - 100 µg / mL. A recuperação média da cafeína foi de 99,89%. A precisão intra e inter-dias (D.P.R.) foi <1,5% e <1,82%, respectivamente, e a precisão (E.R.) ficou na faixa de ± 2,14%. Os resultados, verificados pela validação, mostraram uma técnica adequada a ser aplicada em as amostras avaliadas, além de uma metodologia simples e rápida de execução, com custos mais baixos.

Palavras-chave: Cafeína, Café descafeínado, CLAE-UV, Validação.

Brazilian Journal of health Review

1 INTRODUCTION

Caffeine (1,3,7-trimethylpurine-2,6-dione) (Figure 1) is among the most consumed legal psychostimulants nowadays and is present in many and diverse kinds of foods and beverages[1-2]. This drug has psychoactive properties. It is a slightly dissociative and stimulant drug because of its nonselective antagonist action against adenosine receptors[3-4]. Recent studies have reported that intake of caffeine during pregnancy (plasma concentration estimated by self-reported consumption) is associated with the development of disorders such as obesity and hypertension, besides others[4-6]. Caffeine (CFF) is present in various food products and the most traditional of them is coffee. In Brazil, it is the most popular and indispensable drink in the main meals of the majority of the population[7-8].
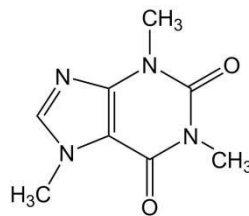

Figure 1. Chemical structure of Caffeine (CFF)

Considering the effects of CFF on the body, the presence of the substance in the diet can interfere in its clinical evolution and even aggravate some situations. As chemical mediators involved in cardiac, renal, circadian cycle changes, anxiety and neural disorders, especially when these disorders are installed[9-10]. In this way, the adoption of decaffeinated products is important to aid in clinical evolution and avoid the aggravations of patients with diseases already installed. And the guarantee of adequate caffeine levels in caffeinated coffee is extremely important due to the frequent presence of the drink in the diet of the population[3,7].

The importance of developing a short, fast and effective method ensures time savings and investment in the analyzes, as well as the reduced use of reagent quantities, reducing waste and contact with toxic substances during the process[11].
The caffeine quantification methodologies with high performance liquid chromatography (HPLC-UV) in the literature present accurate, reliable and fast results[12]. The major problem of analysis is the extraction stage of caffeine, where predominantly long-term studies are described , use of larger quantities of reagents such as magnesium oxide, zinc oxide and some with high toxicity such as lead acetate and dichloromethane[13]. In addition,

the chromatographic reading performed using phosphate buffer, which also reduces the life of the chromatographic column and increases the time of the analysis[12-19].The present work presents simple, rapid and sensitive methods for determination of CFF in commercialized decaffeinated coffee products, using a relatively simple liquid-liquid extraction procedure using in combination with HPLC-UV analysis.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Caffeine reference standard was acquired from the United States Pharmacopoeia (Rockville, MD, USA). HPLC-grade acetonitrile and chloroform was purchased from J.T. Baker(Phillipsburg, NJ, USA). Sulfuric acid, potassium hydroxide was obtained from VETEC (Duque de Caxias, RJ, Brazil).

Water was purified using a MilliQ® system from Millipore (Molsheim, France). Six samples of decaffeinated soluble coffee, purchased at establishments of food products, were selected and the brands of the products were kept confidential for ethical reasons. All products were within the validity, with the indication of clear and visible decaffeinated and with inviolate packages.

2.2 INSTRUMENTATION

High performance liquid chromatography was done using a chromatograph composed of two pumps (LC 20AD), a column oven (CTO 10Avp), an autosampler (SIL 10ADvp), and a system controller (CBM 20A), the LC equipment was connected to UV (SPD 20A), detector Shimadzu (Kyoto, Japan). For sample extraction a Fanem Excelsa II 206 BL centrifuge (São Paulo, Brazil) was used.

2.3 CHROMATOGRAPHY CONDITIONS

For the LC optimization some analytical columns were evaluated. Reversed phase C18 (Gemini 150 x 4.6 mm, 5 μm), reversed phase C18 (ACE, 150 x 4.6 mm, 5μm) and reversed phase monoltic (Phenomenex, Onix 150 x 4.6 mm). The mobile phase was achieved by varying the percentage of organic solvent (methanol or acetonitrile) for a short analytical time, the best compromise between separation efficiency, symmetryand stability of the UV signal.

2.4 PREPARATION OF WORKING SOLUTIONS AND QUALITY CONTROL STANDARDS

The stock solutions of CFF (at 1.0 mg.mL^{-1}) were prepared by dissolving the substance in acetonitrile. The calibration curves for CFF were prepared in mobile phase at concentrations of 10, 25, 50, 75, and 100µg.mL^{-1}. Quality control (QC) samples were also prepared in mobile phase at the following concentrations: 10, 50 and 80µg.mL^{-1} (low, medium and high concentrations). The caffeine content (ppm) of the various samples was calculated by interpolation within the regression equation of the best line of fit. After that the results were presented in percentage on dry basis.

2.5 SAMPLE PREPARATION

The process was started by weighing accurately 0.25 grams of each of the six samples of decaffeinated soluble coffee. Each aliquot was packed in individual beakers, numbered 1 to 6 according to the numbering of the corresponding sample. Then, 5 mL of distilled and deionized water heated at 70 ° C was added, after 0.5 mL of sulfuric acid solution, 2 mol.L^{-1} concentration in each sample and the systems were kept under constant stirring until reach an ambient temperature of 25 ° C. To leave the basic mixtures, 0.5 mL of 5 mol.L^{-1} potassium hydroxide was added keeping each sample under stirring for a few seconds to ensure uniformity of the system. To separate the caffeine from the other coffee components, 5 mL of chloroform was added in each aliquot, shaken and centrifuged at room temperature and 2500 *g* for 5 minutes to optimize the separation. From the separated phases, the aquos portion was removed from each one, where the caffeine has the most affinity and 2 mL of chloroform were added and the same conditions of centrifugation were again submitted. Thereafter, each sample was evaporated to dryness on heating mantle and each residue dissolved in 2 mL of mobile phase.

2.6 METHOD VALIDATION

As recommended by ICH, (2005)[20], the validation characteristics considered in this study were linearity, range, limit of detection (LOD), limit of quantification (LOQ), repeatability and recovery. Five different standards caffeine solution from 10-100µg.mL^{-1} were taken to evaluate the plot of signal as a function of analyte concentration. For precision, the intraday and interday repeatability were performed taking 50µg.mL^{-1} standard solution for 6 determinations For precision (as relative standard deviation (R.S.D.)) and accuracy (as relative error (R.E.)) studies, samples were prepared at with 6

replicates each, and were analysed in the same day (intraday precision and accuracy), and analysed in 2 consecutive days (interday precision and accuracy). The LOQ and LOD were determined by noting the signal to noise ratio comparing measured signals from samples with known concentrations. A signal to noise ratio between 3:1 and 10:1 was considered for LOD and LOQ[20]. Recovery was tested by adding blank samples (decaffeinated coffe) with different caffeine standard concentration and analyzing their content. Then were determined by comparing peak areas of spiked samples with the peak area in solutions prepared with the same nominal concentration.The stability of the solutions and samples was also evaluated during method validation. CFF stock solutions were analysed at $50\mu g.mL^{-1}$ both recently prepared or after 7 hours stored at 4 ºC. The stability of CFF was also evaluated in post-extracted samples keptat room temperature (23 ºC) for 12 or 16 hours, as well as. All samples described above were compared to freshly prepared CFF samples at the same concentration level.

2.7 APPLICATION OF METHOD

The method was applied to a caffeine quantification analyzes, each of the six brands analyzed were analyzed in triplicates. Samples obtained after the caffeine extraction procedure were used to avoid interfering with the procedure for reading the other constituents of the decaffeinated soluble coffee. For the products under study, comply with the decaffeinated specification, in the permitted range, of less than 0.3 g.100g-1, in accordance with Brazilian Resolution RDC No. 277 of September 22, 2005, of the Agência Nacional de Vigilância Sanitária (ANVISA)[21].

2.8 STATISTICAL ANALYSIS

Where applicable, results were expressed as Mean ± Standard Deviation (SD) and analyzed statistically by using Microsoft Excel® 2007. For relevant calculations instrumental software Labsolutions LC Solutions v 1.25 Shimadzu Corporation®.

3 RESULTS AND DISCUSSION

Before the validation parameters were conducted, identification test was conducted to discriminate between caffeine and compounds of closely related structures which are likely to be present. The discrimination procedure was confirmed by obtaining a positive result from coffee products, blank and from standards. No significant background noise or peak interfered or coincided with the peak for caffeine.

3.1. HPLC CONDITIONS

Chromatographic separation was achieved using a ACE C18 column (ACE, Scotland) with 150 x 4,6 mm, 5µm. The mobile phase consisted of acetonitrile and water (20:80, v/v) was filtered, degassed and pumped at a flow rate of 0.8 mL.min^{-1}. The column oven was set at 20 °C and theinjected volume was 20 µL, UV detector set at 285 nm. Retention time for CFF was 4.88 min, and analytical run was 7.0 min (Figure 2).

3.2. METHOD VALIDATION

3.2.1 Specificity

The method demonstrated excellent chromatographic sepecifity with no endogenous or metabolite interferences at the retention times for CFF. Chromatograms of extracted blank containing low (10 µg.mL^{-1}) and high (100µg.mL^{-1}) concentrations indicated good detector response and baseline resolution for CFF. with an analytical run time of 7 min (Figure 2). The carry over test shows not interference between samples of an analysis sequence. The development of caffeine extraction rapidly, without the use of toxic solvents and consequently a cheaper and reproducible analysis.
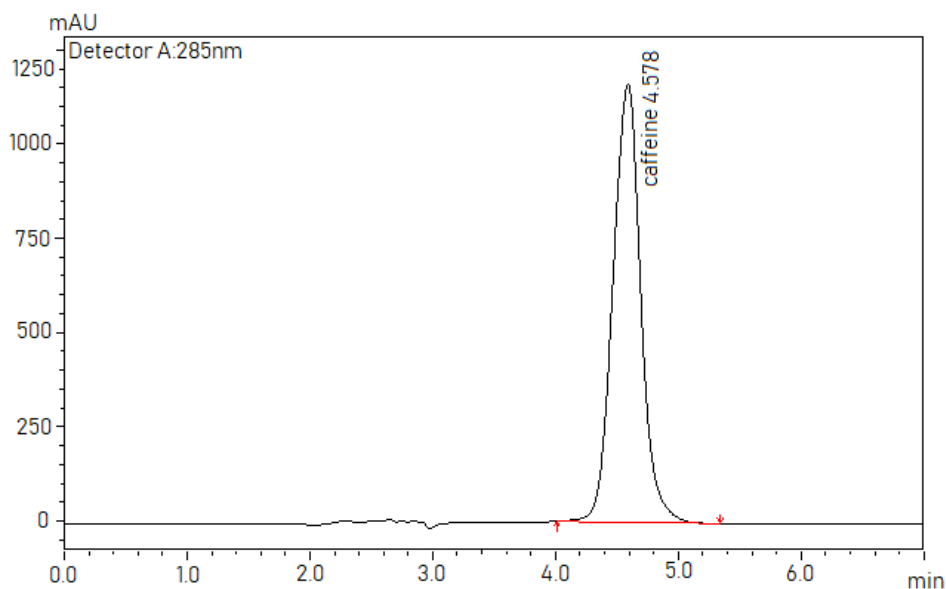

Figure 2. Chromatogram of caffeine in coffee Sample

3.2.2 Linearity, precision, accuracy and recovery

Good linearity was obtained in the concentration range of 10 – 100 µg.mL^{-1} with a mean correlation coefficient of 0.9965 (n= 3 analytical runs, Table 1). Sensitivity of the

method was measured in terms of Limit of detection (LOD) and limit of Quantification (LOQ). The LOD and LOQ were determined by measuring the signal to noise ratio from standard low concentration of analyte comparing with baseline peak of blank sample[20]. The instrumental softwaredetected signal to noise ratio 3:1 for the standard concentration of analyte caffeine at 0.25 µg.mL^{-1}which was out limit of detection for this method. Similarly, LOQ was found to be 0.8 µg.mL^{-1}at which the instrumental software assessed the signal to noise ratio of 10:1 .The recovery of CFF using liquid-liquid extraction with chloroform was 99.89%.

Table 1. Accuracy and precision of Linearity.

Spiked concentration (µg.mL^{-1})	Determinad concentration (µg.mL^{-1}) (Mean ± SD) (n=3)	Precision (%)	Accuracy (%)
10	10.113± 0.255	2.52	101.13
25	24.961± 0.217	0.87	97.84
50	50.352± 0.605	1.20	100.70
75	77.553± 0.481	0.62	103.40
100	103.655± 1.095	1.06	103.65

Matrix effects were investigated by analysis of spike-after-extraction samples with pure standard solutions at the same concentrations and the results is 99.13% for CFF.The repeatability of the method was investigated by performing 6 repeated analysis of standard solution (50 µg.mL^{-1}) on the same day (for intra-day repeatability) and different day for inter day precision. Intra- and inter-day precision and accuracy results (Table 2) gave satisfactory results in that R.S.D. < 1.22 % and R.E. < 2.887%.

Table 2: Precision and accuracy results of validation

Spiked conc. (µg.mL^{-1})	Intraday (µg.mL^{-1}) (n=6)			
	Day 1 Applicator 1 Mean (R.S.D.)	Applicator 2 Mean (R.S.D.)	Day 2 Applicator 1 Mean (R.S.D.)	Applicator 2 Mean (R.S.D.)
50	51.444 (0.48%)	50.395 (1.22%)	50.348 (1.50%)	49.796 (0.64%)
Acc. (R.E.)	2.887	0.790	0.696	0.409

Brazilian Journal of health Review

The accuracy of the method was calculated at concentrations of 25, 50 and 75 $\mu g.mL^{-1}$. The assays were performed in triplicate for each concentration. The results obtained were 99.04, 100.57 and 102.14%, respectively

3.2.3 Stability studies

The stability data of CFF in sample and solution under different temperature, time conditions and freeze-thaw was demonstrated as the calculated concentrations for the controls did not significantly decrease over the course of the study. The stability of CFF was evaluated in post-extracted samples kept at room temperature (23 °C) for 12 or 16 hours, as well as, the stock solutions were analysed after 7 hours stored at 4 °C. The results obtained were 99.56, 98.98 and 100.01%, respectively.

3.3 RESULTS OF CAFFEINE CONTENT IN DIFFERENT COFFEE SAMPLES

Quantitative caffeine analysis in the tested products is described in Table 3. Concentration values were calculated from the caffeine area of each sample. The mean concentration found was 0.1877 $g.100g^{-1}$ ± 10.7. All values obtained were within the permitted range, lower than 0.3 $g.100g^{-1}$, according to Brazilian Resolution RDC No. 277 of September 22, 2005, (ANVISA)[21]. The highest concentration of caffeine found was 0.2388 $g.100g^{-1}$ and the lowest, 0.0946 $g.100g^{-1}$, of samples 1 and 6, respectively.

Table 3: Concentrations of caffeine found in each sample

Caffeine (n=3)		
Test data	Conc. $g.100g^{-1}$	Precision(%)
Sample 1	0.0946	6.935
Sample 2	0.2371	4.103
Sample 3	0.1964	8.937
Sample 4	0.1679	5.566
Sample 5	0.1972	3.768
Sample 6	0.2388	3.480

4 CONCLUSION

We have described a simple, rapid and sensitive HPLC-UV method for the quantitation of CFF in decaffeinated coffee, which showed acceptable precision and adequate sensitivity. The major advantages of this method are the simple sample preparation, the short run time (7 min) for high throughput analysis and good sensibility, which are all important characteristics when dealing with large batches of samples. The

methodology allowed the verification of a fast, less polluting technique with lower analysis costs and satisfactory results in the study of caffeine quantification in decaffeinated coffee.

REFERENCES

Reissig C.J., Strain E.C.& Griffiths R.R. (2009) Caffeinated energy drinks-A growing problem. *Drug Alcohol Depend.* **99**(1–3):1–10

Goldman, R. D. (2013). Caffeinated energy drinks in children. *Can Fam Physician.***59**(9), 947-948.

Fredholm, B. B., Chen, J. F., Cunha, R. A., Svenningsson, P., & Vaugeois, J. M. (2005). Adenosine and brain function. *Int Rev Neurobiol*, **63**(1), 191-270.

Chen, J. F., Eltzschig, H. K., & Fredholm, B. B. (2013). Adenosine receptors as drug targets—what are the challenges?. *Nat Rev Drug discov*, **12**(4), 265.

Klebanoff, M. A., & Keim, S. A. (2015). Maternal serum paraxanthine during pregnancy and offspring body mass index at ages 4 and 7 years. *Epidemiology*, **26**(2), 185-191.

Li, D. K., Ferber, J. R., & Odouli, R. (2015). Maternal caffeine intake during pregnancy and risk of obesity in offspring: a prospective cohort study. *IntJObesity*, **39**(4), 658.

Altimari, L. R., Cyrino, E. S., Zucas, S. M., Okano, A. H., & Burini, R. C. (2008). Cafeína: ergogênico nutricional no esporte. *Revista Brasileira de Ciência e Movimento*, **9**(3), 57-54.

Cazé, R. F., Franco, G. A. M., Porpino, S. K. P., de Souza, A. A., Padilhas, O. P., & Silva, A. S. (2010). Influência da cafeína na resposta pressórica ao exercício aeróbio em sujeitos hipertensos Caffeine influence on blood pressure response to aerobic exercise in hypertensive subjects. *Rev Bras Med Esporte*, **16**(5), 324-328.

Smith, A. (2002). Effects of caffeine on human behavior. *Food Chem Toxicol*, **40**(9), 1243-1255.

Ashihara, H., Sano, H., & Crozier, A. (2008). Caffeine and related purine alkaloids: biosynthesis, catabolism, function and genetic engineering. *Phytochemistry*, **69**(4), 841-856.

Pinto, G. A. T., Freitas, L. G., Machado, Y., & Marinho, P. A. (2015). Avaliação da técnica de imunocromatografia para análise de drogas de abuso no contexto da química forense. *Revista Brasileira de Criminalística*, **4**(3), 28-37.

Brito, N. M., JUNIOR, O. P. D. A., Polese, L., & Ribeiro, M. L. (2003). Validação de métodos analíticos: estratégia e discussão. *Pesticidas: Revista de Ecotoxicologia e Meio Ambiente*, **13**(1), 129-146.

Toci A., Farah A. & Trugo L.C. (2006) *Quim. Nova***29** (5) : 965-971.

Alves, A. B., & Bragagnolo, N. (2002). Determinação simultânea de teobromina, teofilina e cafeína em chás por cromatografia líquida de alta eficiência. *RevBras Cienc Farm*, **38**(2), 237-243.

Moreira, I., Scheel, G. L., Hatumura, P. H., & Scarminio, I. S. (2014). Efeito do solvente na extração de ácidos clorogênicos, cafeína e trigonelina em Coffea arabica. *Quim. Nova*, **37**(1), 39-43.

Walton, H. F., Eiceman, G. A., & Otto, J. L. (1979). Chromatography of xanthines on ion-exchange resins. *JChromatogr A*, **180**(1), 145-156.

De Maria, C. A., & Moreira, R. F. (2007). Cafeína: revisão sobre métodos de análise. *Quim Nova*, **30**(1), 99.

Pokhrel, P., Shrestha, S., Rijal, S. K., & Rai, K. P. (2016). A simple HPLC Method for the Determination of Caffeine Content in Tea and Coffee. *J Food Sci Tech*, **9**, 74-78.

Ferguson, G. K. (1998). Quantitative HPLC analysis of an analgesic/caffeine formulation: determination of caffeine. *J Chem Educ*, **75**(4), 467.

ICH, (2005). Validation of Analytical Procedure, Text and Methodology. International Conference on Harmonization(ICH Q2 (R1). 2005.

Brasil, Resolução RDC nº 277, de 22 de setembro de 2005. ANVISA - Agência Nacional de Vigilância Sanitária.