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Development of an HPLC/ELCD Analytical Method for the Determination of Caffeine, Theophylline, Theobromine and Adenine

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ABSTRACT

An HPLC method with electrochemical detection in the oxidation mode is developed for the determination of caffeine, theophylline, theobromine, and adenine. The method includes a separation step on RP-C8 column using acetonitrile and 0.05M KH_2PO_4 buffer solution in a ratio of 10:90% (v/v) as a mobile phase. Electrochemical detection was conducted on a Glassy Carbon Electrode (GCE) and on a self-made Iodine-coated platinum (Pt-I) electrode. The optimum working potential for GCE used with the Ag/AgCl (3M KCl) as reference electrode was found to be at +1.45 V, whereas that for the Pt-I electrode was found to be at +1.55 V.

The Pt-I electrode shows equal or better sensitivity compared to GCE.

The developed method shows a good applicability (sensitivity, selectivity and reproducibility) for real samples of coffee and tea.

Keywords: HPLC/ELCD, Caffeine, Theophylline, Theobromine, Adenine.

1. INTRODUCTION

The Alkaloids Caffeine (CA), Theobromine (TB) Theophylline (TP), and Adenine (AD) are widely distributed in plant products and beverages and belong to the group of purines (Trugo, Macrae and Dick, 1983). As alkaloids enter the body from food sources, they produce a variety of biological effects; such as stimulation of the central nervous system through changes in the electrical activity of the brain, increasing the gastric acid secretion and diuresis (Graham, 1978). They have been also applied for various disorders in the human being including heart diseases (Clarke and Macrae, 1985). Moreover, they show an effect on the cardiovascular system by relaxation of the smooth muscles of blood vessels and causing an increase of the heart output. They are used also for other disorders such as carcinogenesis, kidney malfunction and asthma (Clarke and Macrae, 1985). It has been proved that there is a strong relationship between these alkaloids when consumed in high concentrations and cancers such as breast cancer (Lubin and Ron, 1990).

Many methods have been published for the analysis of these compounds in foodstuffs and pharmaceuticals. Among these methods are gas chromatography (Wang and Weisheng, 2005; Thomas and Foster, 2004), HPLC (Zhifang and Xiuwen, 2005; Aquino, Wendel, Amorim, Favilla and Nascimento, 2004), and capillary electrophoresis with amperometric detection (Zhang, Hong-Zhen, Wei-Han and Hong-Yuan, 2005; Teshiman, Ogawa, Yamashita and Sakai, 2001; Winrui, Daiging, Qian and Xiaoying, 2000) and HPLC with amperometric detection (Sontag and Kral, 1980; Greenberg and Meyer, 1979; Arai, Kubo, Kinoshita, Nakazawa and Fujita, 1986; Meyer and Ngiruwonsanga, 1996).

For the elimination of the matrix, different techniques, such as liquid-liquid extraction (Muhtadi, El-Hawary and Hifnawy, 1998), column chromatography (Sontag and Kral, 1980) and solid-phase extraction (Meyer and Ngiruwonsanga, 1996; Muhtadi, El-Hawary and Hifnawy, 1998) are necessary to be performed prior to the quantitation step.

The aim of the present study is to develop a simple, selective HPLC-electrochemical detection method for the

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simultaneous determination of the four purine alkaloids in coffee and tea without any matrix separation step. In this method, we successfully use, for the first time, an iodine-coated platinum electrode which shows a higher sensitivity compared to the glassy-carbon electrode.

2. EXPERIMENTAL

Chromatography

The HPLC apparatus comprised a dual head pump model LC 1110 (GBC-Australia) combined with a Rheodyne injector Model 7125 (California, U.S.A.) equipped with a 20 µl loop. A UV-Vis spectrophotometer Model LC-1205 (GBC-Australia) and a Metrohm electrochemical detector (ELCD) Model 656-VA connected to an electrometer Model 641-VA (Metrohm-Switzerland) were used. The ELCD-detector cell was composed of a glassy-carbon electrode or a self-made Pt-I electrode as a working electrode, an Ag/AgCl (3M KCl) as a reference electrode and an integrated gold auxiliary electrode.

The used columns (LiChrosphere RP-C2, RP-C8 and RP-C18) were all of the dimensions 250x4 mm filled with a stationary phase of 5 µm particle size (Knauer, Berlin-Germany). The chromatograms were recorded using a Spectra Physics 4290- integrator (SP-U.S.A.).

The mobile phase was optimized through testing different percentages of acetonitrile and methanol with different aqueous electrolyte solutions at different flow rates where it was found that the mixture of 8% acetonitrile with 92% aqueous potassium dihydrogene phosphate solution (0.05 M, pH 5) at a flow rate of 1.8 ml/min gave the best resolution. This eluent was permanently degassed with helium.

Preparation of the Pt-I Electrode

A platinum electrode was made of polycrystalline platinum as follows: A PTFE sleeve of 4 mm diameter was drilled all through making a 1.90 mm diameter inner barrel. A 2 mm platinum wire was dipped into liquid nitrogen and inserted into the barrel. To ensure the electrical contact with the platinum wire, a drop of mercury was added and the contact with the sleeve was established through a silver wire. Epoxy was added at this end to provide a mechanically stable configuration for the electrode.

The electrode surface was cleaned using a 600 µm emery paper, then it was immersed in chromic acid [5% potassium dichromate in concentrated sulfuric acid] for 30 seconds. After that, the electrode was immersed in an iodine solution (10^{-4} M I^- in 0.1 M NaOH) for 10 minutes. This operation was repeated daily.

Materials and Solvents

Caffeine, theobromine, theophylline and adenine were purchased from Sigma chemicals (Germany) with a 99% purity. Methanol, HPLC-grade (Scharlau Chemie, Spain), acetonitrile, HPLC-grade (May and Baker, England), Potassium dihydrogene phosphate, 99.9% were obtained from Riedel-deHaen (Germany). Water used for the mobile phase was freshly prepared and triply distilled, where potassium permanganate (50 mg/L) was added to the second distillation step and, finally, the water was passed through a LiChrosorb RP-C18 column (250 x 4 mm, 63 µm particle size).

Standard Solutions

A 1000 µg/ml stock solution of each of the four compounds were prepared by dissolving 10.0 mg in 10.0 ml water using the ultra-sonic bath. These stock solutions were kept at +5ºC in the refrigerator and are stable for two weeks. The working standard solution for each compound and the standard mixture solution both were prepared by appropriate dilution with the eluent.

3. RESULTS

Optimization of Separation System Using UV-Detector

The working parameters for the separation system were optimized using first the UV-spectrophotometer at 245 nm. The best separation was achieved on a RP-C18 column using a mixture of 8:92 % (v/v) acetonitrile-aqueous buffer [0.05 M KH_2PO_4, pH 5] as an eluent at 1.8 ml/min flow rate. Figure (1) shows the separation of the four compounds [adenine 1 ppm, theobromine 2 ppm, theophylline 2 ppm, and caffeine 3 ppm] using these conditions.

Optimization of Separation Systems Using ELCD with GCE and Pt-I Electrodes

The same optimized chromatographic conditions found for UV-detector were used in connection with the electrochemical detector using the Glassy-Carbon Electrode (GCE) and the self-made iodine-coated platinum electrode (Pt-I). The best working potential was found at +1.45 V at a detector sensitivity of 50 nA for

GCE and 5 nA for Pt-I electrode. Keeping the parameters of the ELCD constant, the chromatographic parameters were further optimized using the Pt-I electrode. The best separation (highest resolution and sensitivity) was found at the following parameters: 10:90% (v/v) acetonitrile-aqueous buffer solution [0.05M KH_2PO_4, pH 5] as eluent at 1.8 ml/min flow rate. RP-C8 column [250 x 4 mm, 5 µm particle size] gave a good separation with lower retention times. Figure (2) shows the separation of a mixture of the four compounds [adenine 0.2 ppm, theobromine 0.4 ppm, theophylline 0.4 ppm and caffeine1 ppm] using these optimized conditions on both electrodes.

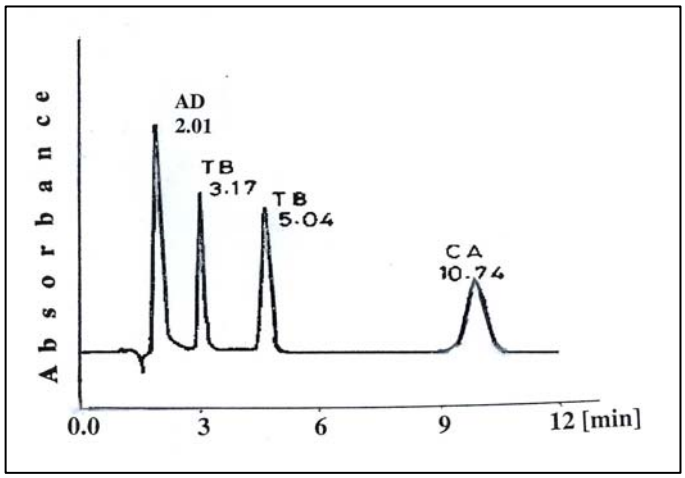

Figure 1: Optimized Separation using UV-detection (λ= 254 nm), using RP-C18 column (250 x 4 mm, 5 µm) with acetonitrile (CAN)/ aqueous buffer (8:92%) at a flow rate of 1.8 ml/min.

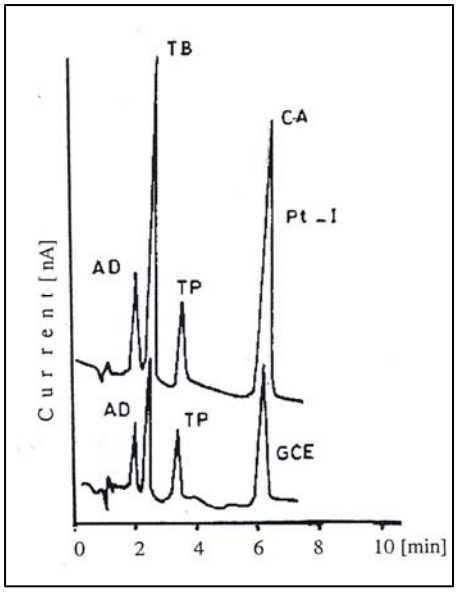

Figure 2: Optimized separation using Pt-I and GCE electrodes at +1.45V, RP-C8 column (250x 4 mm, 5 µm) with ACN/aqueous buffer (8:92%) at 1.8 ml/min.

4. METHOD VALIDATION

Detection Limits

Limit of detection was determined by using the signal-to-noise ratio of 3:1. The average of five runs of the standard mixture of the four compounds was calculated and the results are shown in Table (1).

Linearity and Linear Range

Table (2) shows the results of the calibration curves and range of the four compounds. Linearity was characterized as the regression coefficient of the test results of peak heights vs. analytic concentration relationship.

Table 1: Detection limits.

Compound	Detection Limits [ng]	
	Using GC-electrode	Using Pt-I-electrode
Adenine	1	0.3
Theobromine	10	0.5
Theophylline	0.3	0.4
Caffeine	18	1.3

Precision

These were determined by the injection of a standard mixture containing the four compounds five times. The Coefficient of Variation (CV) for the peak heights and for the retention times of the four compounds using the glassy carbon electrode and the iodine-coated platinum electrode are shown in Table (3).

Recoveries

The whole method, including the extraction step described under the application, was tested for its efficiency using spiked water samples in a range from 6 to 500 ng/ ml. The calculated recoveries for all the four compounds ranged between 85% and 98%.

Interferences

Figure (3) shows a chromatogram of a Black Yemini Coffee sample prepared as shown in the next paragraph. From this chromatogram it is clear that there are almost no interferences and the peaks appeared are only those of the four compounds and that of the solvent (first peak).

Table (2): Linearity and linear range using the ELCD/Pt-I System.

Compound	Concentration linear range [ng]	Intercept	Slope	Correl. coeff. r
Adenine	6-26	17.895	0.663	0.9945
Theobromine	40-800	7.484	0.062	0.9998
Theophylline	16-60	11.715	0.318	0.9977
Caffeine	120-500	0.970	0.059	0.9985

Table (3): Comparison between the Coefficients of Variation (CV) (precision) for the Glassy-Carbon-Electrode (GCE) and the Pt-I electrode.

	CV (%) using GCE				CV (%) using Pt-I			
	AD	TB	TP	CA	AD	TB	TP	CA
Peak Height	0.24	0.65	0.11	0.14	0.14	0.20	0.26	0.09
Retention Time	0.31	0.28	0.32	0.42	0.25	0.25	0.21	0.23

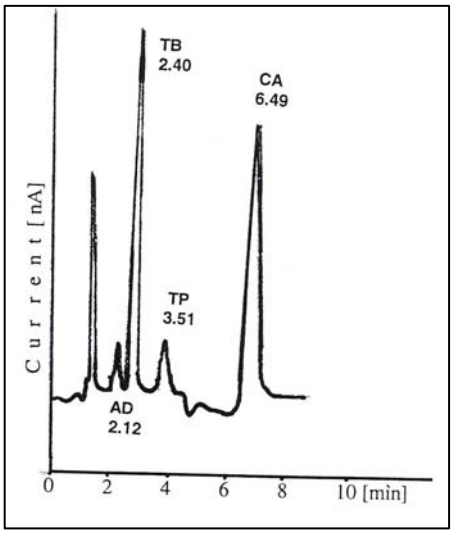

Figure (3): Chromatogram of a brewed Black Yemini Coffee sample using Pt-I electrode. Other Conditions are as in Fig. 2.

Application on Coffee and Tea

Different coffee and tea samples were gathered from the local market, and classified according to the roasting grade (green, blonde or black) as shown in Table (4).

The extraction of the coffee samples was done by brewing 5.0 g with 100 ml triply distilled water for 10 min. The sample was then filtered and 1.0 ml of the extract was diluted in 25 ml volumetric flask. The tea samples were extracted by brewing one bag or 0.3 g in 100 ml triply distilled water for 5 min and filtered. Both extracts were analyzed without further cleanup using the Pt-I electrode through the injection of 20 µl onto the HPLC column under the above mentioned conditions. The results are shown in Table (4).

Table (4): Concentration of AD, TB, TP, and CA in coffee and tea samples using the Iodine coated platinum electrode.

Sample	AD (µg/g)	TB (µg/g)	TP (µg/g)	CA (µg/g)
Yemeni Black Coffee	3.5 ± 0.8	15.7 ± 1.0	-	385.7 ± 2.7
Yemeni Blonde Coffee	5.2 ± 1.3	20.9 ± 1.4	-	399.7 ± 2.8
Yemeni Green Coffee	17.5 ± 4.1	87.3 ± 5.7	-	488.7 ± 3.4
Brazilian Black Coffee	1.8 ± 0.4	29.7 ± 1.9	-	300.2 ± 2.1
Brazilian Blonde Coffee	10.5 ± 2.5	52.3 ± 3.4	-	343.8 ± 2.4
Brazilian Green Coffee	34.9± 8.5	157.1 ± 10.2	-	349.0 ± 2.5
Saudi Blonde Coffee	22.7 ± 5.5	104.7 ± 6.8	-	315.9 ± 2.2
Nescafe	15.0 ± 3.6	575.0 ± 37.4	67.5 ± 4.9	1550 ± 1.0
Ceylon Tea	10.0 ± 2.4	200.0 ± 13.0	53.3 ± 3.9	2533 ± 17.6
Lipton Tea Bag	10.0 ± 2.5	80.0 ± 5.2	-	795.0 ± 5.5

5. CONCLUSIONS

These results show that the method is easy to handle because there is no cleanup step. The method is also selective, accurate and precise.

In addition to the known high sensitivity and selectivity of the ELCD using the GCE, Figure (2) shows that the new self-made Pt-I electrode is more sensitive than the GCE. The results obtained demonstrate the applicability of the suggested method for the analysis of the four alkaloids in different food stuffs.

Also, the results show that the concentrations of the studied compounds in coffee are inversely proportional to roasting degree of the coffee. This could be due to the sublimation and/or degradation of the alkaloids during the roasting process. It is interesting to notice that the concentration of caffeine and theobromine in Nescafe and tea samples are much higher than in the coffee samples. Theophylline was found only in Nescafe and in tea samples.

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تطوير طريقة تحليلية (HPLC) تستخدم الكروماتوغرافيا السائلة عالية الأداء من أجل تحليل كل من الكفائين والثيوفيللين والثيوبرومين والأدنين

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ملخـص

طورت طريقة كروماتوغرافيا سائلة عالية الأداء (HPLC) مع المكشاف الكهروكيميائي لتحليل كل من الكفائين والثيوفيللين والثيوبرومين والأدنين.

تتكون الطريقة من خطوة استخلاص وخطوة فصل على عمود RP-C8 باستخدام طور متحرك مكون من الاسيتونيترايل مع محلول منظم مكون من KH_2P0_4 0.05M وذلك بنسبة 90:10% (حجم/حجم).

جرى الكشف الكهروكيميائي باستخدام القطب الكربوني الزجاجي (GCE) وكذلك باستخدام قطب مصنع محليا وهو القطب البلاتيني المغطى باليود (Pt-I). وجد فرق الجهد الأمثل لتشغيل القطب الكربوني الزجاجي عند 1.45+ فولت، بينما وجد فرق الجهد الأمثل لتشغيل القطب Pt-I عند 1.55+ فولت.

كما وجد أن القطب (Pt-I) ذو حساسية تساوي حساسية القطب الكربوني الزجاجي أو أحسن منها.

أعطت هذه الطريقة الحديثة نتائج جيدة (من ناحية الحساسية والانتقائية والتكرارية) عند استخدامها لتحليل عينات حقيقية من القهوة والشاي. وكانت حدود الكشف التي تم الحصول عليها لمواد الكفائين والثيوفيللين والثيوبرومين والأدنين هي 0.3 نانوغرام و0.5 نانوغرام و0.4 نانوغرام و1.3 نانوغرام على التوالي وذلك باستخدام القطب الجديد (Pt-I).

الكلمـات الدالـة: الكروماتوغرافيا السائلة عالية الأداء (HPLC/ELCD)، كفائين، ثيوفيللين، ثيوبرومين، أدنين.

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