Determination of Theobromine, Theophylline, and Caffeine in by-Products of Cupuacu and Cacao Seeds by High-Performance Liquid Chromatography

F. Lo Coco[1,*], F. Lanuzza[2], G. Micali[2], and **G. Cappellano[2]**

[1]Dipartimento di Scienze Economiche, Area Ambientale ed Agroalimentare, Università degli Studi di Udine, Via Tomadini 30/A, 33100 Udine, Italy and [2]Dipartimento di Studi su Risorse, Impresa, Ambiente e Metodologie Quantitative, Università degli Studi di Messina, Piazza S. Pugliatti 1, 98122 Messina, Italy.

Abstract

Theobromine, theophylline, and caffeine are determined simultaneously by a rapid and selective reversed-phase high-performance liquid chromatography (HPLC) method with UV detection in by-products of cupuacu and cacao seeds. The determination is carried out in the raw and roasted ground cupuacu seeds and in the corresponding powders obtained after pressure treatment. The by-products of both cupuacu seeds and cacao seeds are obtained under the same technological conditions. The HPLC method uses isocratic elution with a mobile phase of methanol–water–acetic acid (80:19:1) (v/v) at a flow rate of 1 mL/min and UV absorbance detection at 275 nm. Total elution time for these analytes is less than 10 min, and the detection limit for all analytes is 0.1 mg/g. The amounts of theobromine and caffeine found in all the cupuacu samples are one or more orders of magnitude lower than those from cacao. Theophylline is found in all cacao samples except for the roasted ground paste, and it is only found in the roasted ground paste in the cupuacu samples.

Introduction

Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) are natural alkaloids that are present in tea leaves, coffee, and cacao seeds and, therefore, in the food and beverages made from them (1). These alkaloids are contained in a variety of pharmaceutical products and drugs because they possess the following properties: to stimulate the central nervous system, to induce gastric secretions, and to act as a diuretic (2,3). Studies have also been done on these alkaloids to assess any antioxidant properties (4).

Caffeine stimulates the central nervous system, cardiac muscle, the respiratory system, and gastric secretion. The recommended daily dose is 200 mg/day (5). A dose of 10 g is lethal, which is equivalent to about 100 cups of coffee (6). Recent epidemiological studies have seen an association between consumption of caffeine and risk of miscarriage (7). 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 (1,8). This substance can induce addiction and anxiety (9). A number of sporting organizations consider caffeine to be one of the prohibited nervous system stimulants, given that it heightens performance and diminishes fatigue. However, as it is part of a normal daily diet, a concentration level of 12 µg/L in urine is permitted (10–12). Caffeine is used in cola-based drinks in concentrations of approximately 0.1 mg/mL, and drink manufacturers 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 (13).

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 (1).

Various 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 (1,5,6,8,10,14). Ionic chromatography (15) and capillary electrophoresis (16) are also used as well as gas chromatography coupled with mass spectrometry prior to solid-phase extraction (SPE) (17).

The presence of caffeine, theobromine, and theophylline in the paste obtained in cupuacu derivates was studied as part of the research program to characterize the fruit of the cupuacu (*Theobroma grandiflorum*) (18,19). This is a plant from the Amazon and is from the same family as cacao (*Theobroma cacao*). The xanthines were determined simultaneously using reversed-phase HPLC with spectrophotometric detection. The results from the derivates of cupuacu seeds were compared with the ones obtained from the corresponding derivates of cacao seeds. The derivates of the cupuacu seeds and the cacao seeds were obtained under the same technological conditions.

* Author to whom correspondence should be addressed: email filippo.lococo@uniud.it.

Experimental

Samples

Samples were analyzed for both cupuacu and cacao. The paste was obtained from the ground seeds (both raw and roasted), and the related powder was obtained from the paste after pressing at a specific pressure and temperature where the fat content is influenced by the technological conditions adopted. The derivates of both the cupuacu and the cacao were obtained industrially under the same technological conditions.

Chemicals

Caffeine, theobromine, and theophylline (all 99.9% pure) were obtained from Sigma-Aldrich (Milan, Italy). HPLC-grade methanol, analytical-grade acetic acid, aqueous solutions Carrez I [24% (w/v) zinc acetate and 3% acetic acid], and Carrez II [15% (w/v) potassium ferrocyanide] were obtained from Merck (Darmstadt, Germany). A certified T0329 matrix, for caffeine only, of instant coffee powder by Defra (Sand Hutton, UK) was used. Ultrapure water was obtained through a Milli-Q water purification system (Millipore, Bedford, MA).

Equipment

A Thermo Finningan P 2000 series liquid chromatograph (Palo Alto, CA) and a UV–vis detector Thermo Finningan 200 series were used. Data were collected using Peak-96 software (Hewlett-Packard, Palo Alto, CA). A Rheodyne valve was used for injection (Cotati, CA) with a 20-µL loop.

Sample preparation

One gram of the sample was accurately weighed in a conical flask of appropriate volume, 70 mL of water was added, brought to boil, and left to boil for 60 min within a refrigeration system; the sample was cooled and quantitatively transferred into a 100-mL graduated flask; 5 mL of Carrez I solution was added, and, after shaking, 5 mL of Carrez II solution was added and brought up to volume with water. The sample solution was passed through a pleated filter, purified on an SPE Strata C18-U Phenomenex column (Chemtek Analytica, Anzola, Italy), activated with methyl alcohol, taken through a 0.2-µm cellulose filter, and injected into the chromatographic system.

Chromatographic analysis

Components were separated using a Supelcosil LC-18 column (250 × 4.6 mm, 5 µm, Supelco, Sigma-Aldrich, Milan, Italy) and a Supelguard LC-18 precolumn (20 × 2.1 mm, Supelco). Analyses were carried out with isocratic elution at room temperature, injecting 20 µL of solution, using methanol–water–acetic acid (80:19:1) (v/v) as the mobile phase at a flow rate of 1 mL/min. UV–vis spectrophotometric detection was done at 275 nm.

Standard solutions for quantitative determination were prepared with the following concentrations: 1000 µg/mL for theobromine and 100 µg/mL for both theophylline and caffeine. The solutions were filtered through a 0.2-µm cellulose filter and stored in the dark at 4°C. Calibrating solutions were prepared by diluting standard solutions to the following concentration intervals: 1–1000 µg/mL for theobromine, 1–50 µg/mL for theophylline, and 1–100 µg/mL for caffeine.

Results and Discussion

HPLC was used with spectrophotometric detection for the determination of theobromine, theophylline, and caffeine in derivates of cupuacu and cacao seeds obtained industrially under the same technological conditions.

Figure 1 shows the chromatograms of a standard mix and the chromatograms obtained from roasted cacao paste and roasted cupuacu paste.

Qualitative analysis was performed by comparing, for each analyte, the chromatographic behavior and the UV spectra (recorded using the stop flow technique) with that of the standards analyzed under the same chromatographic conditions. Quantitative determination was done by linear regression plots using the concentration intervals indicated in the chromatographic analysis section. Good linearity for the concentration intervals examined as exhibited by the equations and the coefficients of determination for the calibration plots $y = (1.445)x$ and $R^2 = 0.9995$ ($n = 3$) ($t_{exp} > t_{tab}$; 63.2 > 4.3, where t_{exp} is the experimental and t_{tab} the tabulated student's coefficients) for theobromine, $y = (1.214)x$ and $R^2 = 0.9997$ ($n = 3$) ($t_{exp} > t_{tab}$; 81.3 > 4.3) for theophylline, and $y = (1.209)x$ and $R^2 = 0.9997$ ($n = 3$) ($t_{exp} > t_{tab}$; 81.6 > 4.3) for caffeine (20), were observed. Repeatability was evaluated by independently analyzing the same sample six times and verifying that the analytical results were normally distributed (Shapiro-Wilk test) and that there were no anomalous data (Dixon's test) (21, 22); the relative standard deviation was 1.2% for theobromine, 1.4% for theophylline, and

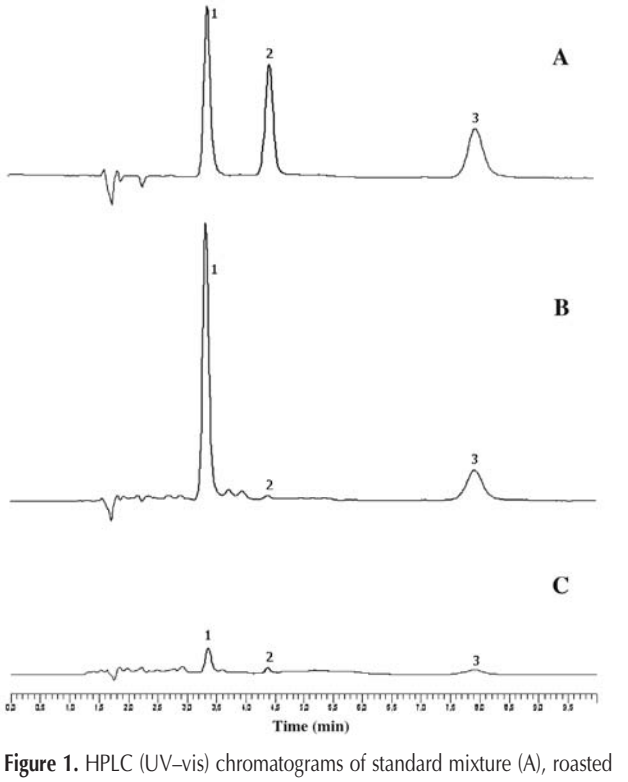

Figure 1. HPLC (UV–vis) chromatograms of standard mixture (A), roasted cacao paste (B), and roasted cupuacu paste (C). Peaks numbers are: theobromine, 1; theophylline, 2; and caffeine, 3. Chromatographic conditions are described in the Chromatographic analysis section.

Table I. Concentration of Xanthines in Industrially Obtained Samples From the Seeds of Cupuacu and Cacao

	Theobromine mg/g*		Theophylline mg/g*		Caffeine mg/g*	
Samples	Cacao	Cupuacu	Cacao	Cupuacu	Cacao	Cupuacu
Raw ground paste	33.00	1.00	0.20	–	5.60	0.50
Roasted ground paste	36.00	1.00	–	0.30	3.30	0.50
Powder from raw ground paste	42.00	0.05	0.20	–	6.00	0.50
Powder from roasted ground paste	59.50	0.25	2.00	–	8.00	1.00

* Average of three analysis.

1.3% for caffeine. The analytical results relating to caffeine content for the certified matrix enable accuracy to be evaluated for both sample treatment as well as analytical procedure by comparing the results obtained (35.4 ± 0.5 mg/g; average ± SD) with those certified (36.0 ± 2.4 mg/g; average ± SD) and these agree well ($t_{exp} < t_{tab}$; 0.2 < 4.3, n = 6, p = 0.95) (23). Detection limits under the conditions adopted were 0.1 mg/g for all three alkaloids, using a signal-to-noise ratio of 3.

The proposed method was employed for the analysis of four cupuacu seed samples and four corresponding cacao seed samples obtained industrially. The results are shown in Table I.

As the data in the Table I shows, the concentrations found for theobromine and caffeine in all the cupuacu samples are one or more orders of magnitude less than those from cacao. Theophylline was found in all the cacao samples with the exception of the roasted ground paste, whereas it was only found in the roasted ground paste for the cupuacu samples, and its concentration is comparable with that of the cacao raw ground paste and cacao poweder of raw ground paste.

Conclusion

The analytical procedure described allows the separation and accurate determination of the three xanthines in by products of cupuacu and of cacao. The LC analysis was carried out under isocratic conditions and was completed in less than 10 minutes.

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