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Determination of Theobromine and Caffeine in *Theobroma cacao* Husk from Ethanolic Extract by GC-MS after CC Separation

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Abstract

After CC separation of 21 samples from *Theobroma cacao* Husk was analyzed by GC-MS, from ethanolic extract at 90% was recovered a solid residue of 1 g after several days on the table of the Lab. This main residue (300 mg) was analyzed after successive separations with mixtures of $CHCl_3$: MeOH (7:3) and $CHCl_3$: MeOH (5:5) in CC using Sílica Gel G 60-120 Mesh. All samples were analyzed by TLC on silica gel with fluorescent indicator 254 nm on aluminum cards (layer thickness 0.2 mm) (10 × 20 cm) using $CHCl_3$: MeOH: n-propanol: water (5:6:1:4) as eluent (v/v/v). The presence of Caffeine (CF) and Theobromine (TB) was confirmed after several analyses of the samples by their characteristic fragmentation pattern but nevertheless was found the presence of Theophylline.

Subject Areas

Bioengineering, Biotechnology

Keywords

Theobroma cacao, Caffeine, Theobromine, GC-MS, CC, TLC

1. Introduction

Total production of cacao bean is estimated worldwide to be approximately 4.0 million tons in 2013, with a value about $12 billion. Residues or wastes from the cacao processing industry consist of cacao pod shell, husk, pulp/mucilage, and

hull, which account for a high proportion, approximately 85% by fresh weight of total cacao pod mass, in which the annual worldwide amount of cacao pod husk is estimated to be about 55 million tons, which is equal to 13 times the total amount of cacao bean. Therefore, cacao pod husk needs to be exploited to produce high-value-added products and this waste has been considered as an abundant, inexpensive, and renewable source of caffeine and theobromine, which exhibits stimulatory effects on the central nervous, gastrointestinal, cardiovascular, renal, and respiratory systems [1].

Native to lowland rainforests of the Amazon and Orinoco river basins, cacao is grown commercially in the New World tropics as well as western Africa and tropical Asia. Its seeds, called cocoa beans, are processed into cocoa powder, cocoa butter, and chocolate [2].

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The consumption of cocoa/chocolate 1) increases plasma antioxidant capacity, 2) diminishes platelet function and inflammation, and 3) decreases diastolic and systolic arterial pressures. Data currently available indicate that daily consumption of cocoa-rich chocolate (rich in polyphenols) may at least partially lower cardiovascular disease risk. Further studies are required in order to establish the bioavailability and mechanisms of action of bioactive compounds in chocolate [4].

Methylxanthines, theobromine and caffeine are used as analgesics, diet aids, and cold/flu remedies in numerous popular carbonated drinks. They are also the main compounds found in tea, coffee, sodas, chocolate, and various energy drinks. More than 120,000 tons of caffeine is consumed worldwide annually [5].

The analysis of theobromine and caffeine in foods, biological fluids, environmental samples, plants, and water, was provided with different instrumental methods, such as high-performance liquid chromatography (HPLC) [6], gas chromatography-mass spectrometry-flame ionization detection (GC-MS-FID) [7], Fourier transform-infrared spectrophotometry (FT-IR) [8], near-infrared spectroscopy [9], UV-Vis spectrophotometry [10], FT-Raman spectrometry [11], and capillary electrophoresis (CE) [12].

The aim of this research was to determine the presence of theobromine, caffeine and theophylline in a solid sample from ethanolic extracts at 90% from *Theobroma cacao* husk after carried out the insoluble solid by CC and running by CG-MS the 21 samples recovered after CC elution.

2. Materials and Methods

2.1. Sample Collection and Processing:

The sample was the husk of cocoa bean after its separation from the fruits. It was

supplied in 2018 by the Chocolate Factory located in Baracoa, Guantánamo Province, Cuba. After the collection the husks were packet in nylon bags without elimination of foreign matters. The material was grounded in a high-speed hammer mill. The sample keeps its brown color (Figure 1) and a very nice chocolate's smell.

2.2. Extract Preparation

The extracts were prepared with the ground material (60 g) without screen extracted in a Soxhlet apparatus with 675 mL of ethanol at 90% for 20 hours. The ethanolic extracts were concentrated and evaporated under vacuum to 200 mL at 120 rpm, a temperature of 70˚C and 500 mbar.

2.3. CC Separation of the Samples

After extraction the ethanolic extract was kept on the Lab table during few days. A solid yellowish-green residue (1 g) appeared on the bottom of the recipient and was recovered by decantation (Figure 2).

300 mg of this solid was then passed thought a CC (31.5 cm × 1.5 cm ϕ) (Figure 3) using as stationary phase Sílica Gel G 60 - 120 Mesh (Merck) and as mobile phase 25 mL of a mixture of $CHCl_3$: MeOH (7:3). After that point, was added successively, 20 mL of pure MeOH, 30 mL of $CHCl_3$: MeOH (7:3); 10 mL of MeOH and finally, 20 ML of $CHCl_3$: MeOH (5:5). The column was cleaned first with chloroform.

Figure 1. Toasted cacao beans, husk and powered drug.

Figure 2. Solid residue from ethanolic extract of cacao husk.

Figure 3. Chromatography column.

2.4. Procedures, Instrumentation and Parameters

The samples were subjected to chromatographic analysis in equipment GC/MS; brand Shimadzu QP2010, equipped with a splitter split/splitless. With a BP5 (30 m × 0.25 mm × 0.25 microns) capillary column under the following chromatographic conditions: Helium gas carrier obtained by electron impact fragments to a power of 70 eV rate of 1.2 mL/min, 1:50 split flow and the volume of injected sample of 1 µL. Programmed oven temperature: initial temperature was 70˚C with a heating ramp of 10˚C/min to 300˚C and remained stable at this temperature for 10 minutes. Subsequently the temperature was increased at a rate of 10˚C/minute to 300˚C for a total time of 78 minutes with an injector temperature 250˚C and the interface temperature 300˚C. The compounds were analyzed using GC/MS NIST21 and NIST107 library and having into account the results obtained after phytochemical screening according with González *et al*., 2017. Silylation agent was *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), CAS 25561-30-2, Lot: 0901-1 Macherey-Nagel GmbH & C. KG.

3. Results and Discussion

Twenty-one samples were recovered from the CC after solid insoluble sample separation (Figure 4). Five fractions (**1 - 5**) were obtained using a mixture of $CHCl_3$: MeOH (7:3). Successively were added pure MeOH (**A-D**), alternating with $CHCl_3$: MeOH (7:3) (**6 - 11**), MeOH (**E-F**) and $CHCl_3$: MeOH (5:5) (**12 - 15**). In general, were taken 21 samples of 5 mL; 15 of them from the mixture of $CHCl_3$: MeOH and six from pure MeOH.

The twenty-one samples after running by CG-MS showed a general chromatogram as shown in Figure 5. The most prominent peaks are between 25 and 40 minutes of retention time with some few exceptions.

The automatically comparison of the results with NIST 21 and NIST 107 Libraries allow us to tentatively suggest the presence of caffeine and theobromine in all samples but with different retention times like is presented in Table 1. In all cases caffeine appear firstly than theobromine. The first Methylxanthine was

Figure 4. Collected samples from CC after insoluble solid separation.

Table 1. Retention times of caffeine and theobromine in each sample.

Sample	Caffeine	Theobromine	Manually predominant compound
1	29.490	29.695	Caffeine
2	29.245	29.695	Theobromine
3	29.235	29.865	Theobromine
4	29.230	29.820	Theobromine
5	29.270	29.665	Theobromine
6	29.265	29.695	Theobromine
7	29.250	29.705	Theobromine
8	29.260	29.690	Theobromine
9	29.260	29.880	Theobromine
10	29.255	29.710	Theobromine
11	29.260	29.840	Theobromine
12	28.250	29.890	Theobromine
13	29.250	30.005	Theobromine
14	29.240	29.905	Theobromine
15	29.235	29.800	Theobromine
A	29.260	29.775	Uncertain
B	29.265	29.610	Uncertain
C	29.240	29.750	Theobromine
D	29.245	29.710	Theobromine
E	29.260	29.625	Theobromine
F	29.245	29.915	Theobromine

between 29.230 min and 29.270 min of retention time, while the second one was between 29.610 and 30.005 min of retention time.

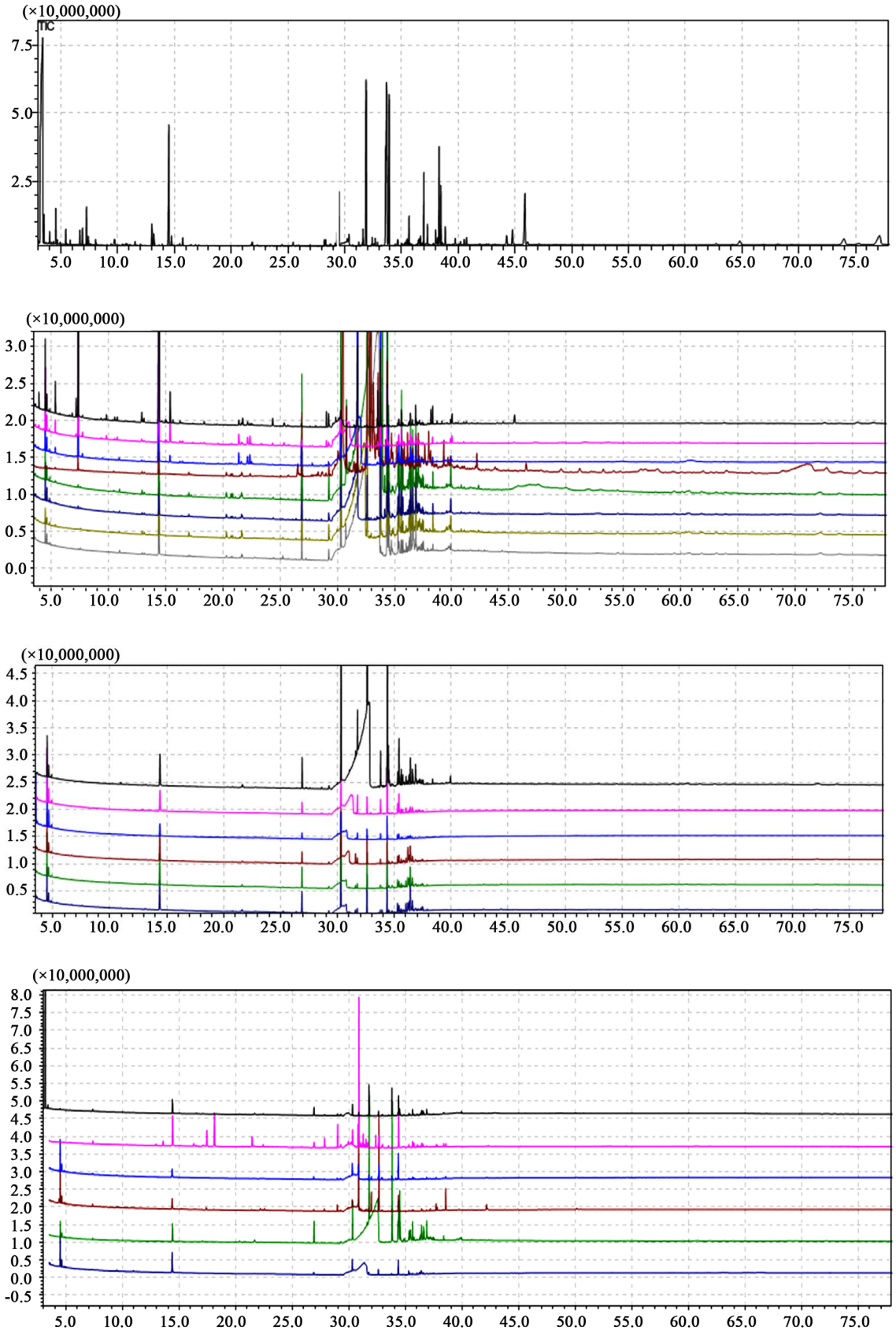

Figure 5. Current chromatograms of the samples (from top to down: 1; 2 - 9; 10 - 15; A-F).

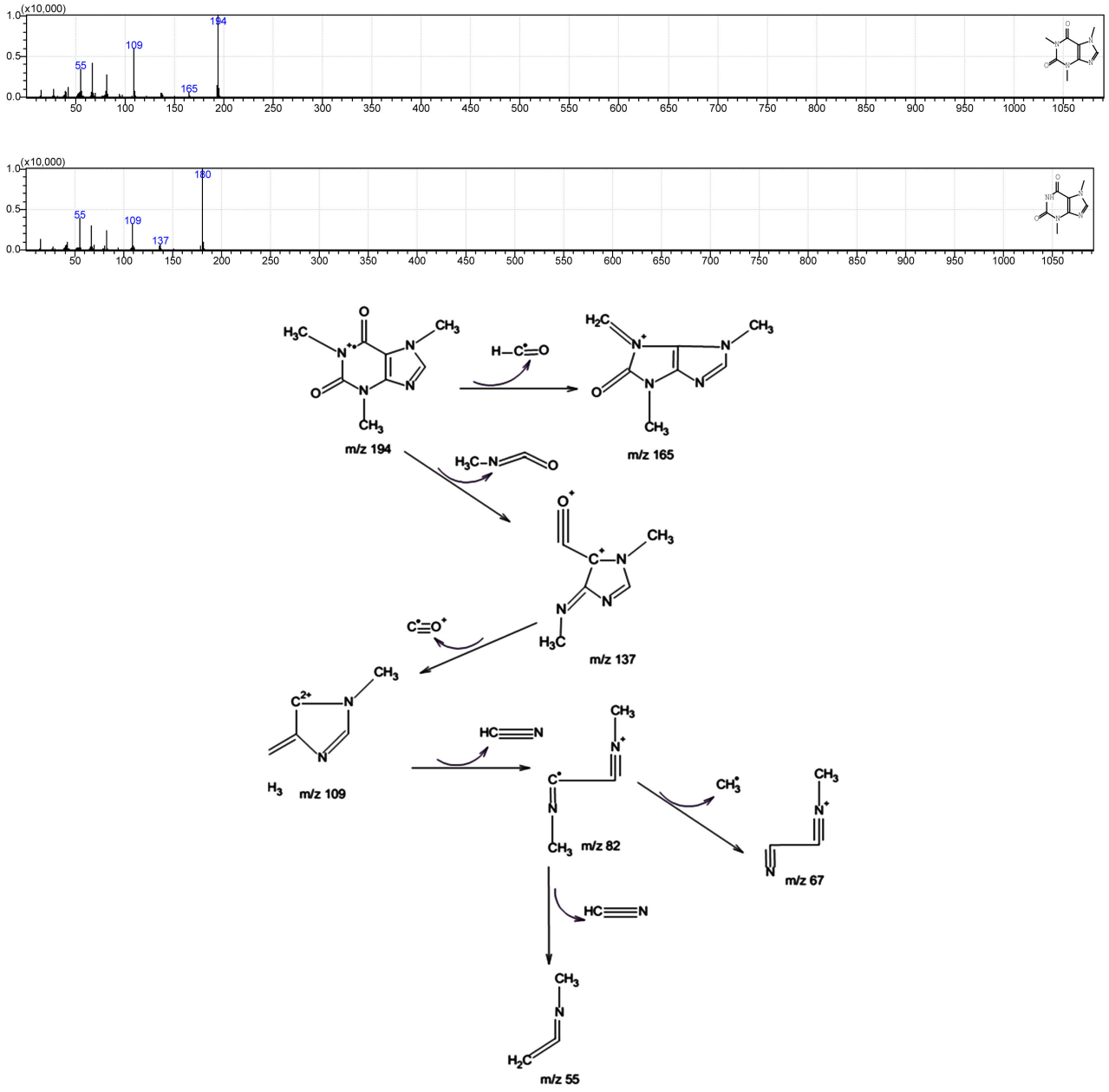

Figure 6. Fragmentation pattern of theobromine and caffeine.

Manually registration of each compound showed a big difference, because using this kind of selective search each sample had another behavior. Sample 1 showed the presence of caffeine at 29.485 minutes of retention time, according to NIST 107 Library with a coincidence of 91% - 93%, while in sample 2 the coincidence was only 65% according to NIST 21 Library to theobromine as a predominant compound.

No theophylline was detected in this research. According to Vázquez-Ovando in 2016 [13], the main alkaloid in cacao is theobromine, but both compounds can coexist because of the presence of the theobromine sintase enzyme. Accumulation of purine alkaloids is dependent on the presence of substrate specificity

of N-metil-transferase.

Using LC–ESI–MS, according to [5], the researchers found that both compound (caffeine and theobromine) had the same fragmentation pattern in the peak m/z 137. As discussed in [14], using different extraction methods and HPLC, reported that both alkaloids had the same fragmentation pattern and declare that is possible to find out the superposition of theobromine, theophylline and caffeine as a mixture.

Figure 6 shows the fragmentation pattern of both chemical components and the fragmentation pattern of caffeine according to [15] and the data from our research group in this investigation.

4. Conclusion

This study was an exploratory and valuable assessment for further studies. The findings from this study indicated that cacao pod husk had high contents of caffeine and theobromine, but low or none contents of theophylline. Therefore, the use of these extraction and purification conditions for effective exploitation of theobromine and caffeine from cacao pod husk is a promising and sustainable trend. In further studies, it is necessary to determine the biological variability of theobromine and caffeine in cacao pod husk samples, as well as produce theobromine-caffeine enriched powder and evaluate its biological activity in various models for potential application in the medical, nutraceutical and pharmaceutical industries.

Conflicts of Interest

The authors declare no conflict of interest.

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