To the Graduate Council:

I am submitting herewith a dissertation written by José Merched Chaar entitled "Extraction of Caffeine from Seed of Guaraná Fruit." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Technology and Science.

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EXTRACTION OF CAFFEINE

FROM SEED OF

GUARANÁ FRUIT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

José Merched Chaar

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DEDICATION

This dissertation is specially dedicated to my wife Silvia O. Chaar for her love, support, and encouragement.

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ABSTRACT

The objectives of this research were to determine the most appropriate treatments and conditions for extracting caffeine from guaraná (*Paulinia cupana* var. *sorbilis* (Mart.) Ducke) seed and to determine the most effective solvents to accomplish the extraction.

Seeds of guaraná, a Brazilian fruit, were prepared for extraction of caffeine. The procedures for preparation included: grinding seeds into a meal, rehydrating with acid or alkaline solutions, and steaming. Extraction was accomplished with single-stage (solvent was not replaced with fresh solvent during the extraction period) or multiple-stage (solvent was replaced with fresh solvent after specified periods of time) processes and with or without agitation. The solvents were ethanol-water mixtures, methylene chloride, and ethyl acetate.

Seeds of the two drying methods, traditional (TD) and solar (SD), were analyzed. TD seeds contained the greater amount of all proximate components, except carbohydrates, and the minerals: calcium, potassium, cooper, and iron. TD and SD seeds contained 4.07% and 4.40% caffeine, respectively. Fifteen volatile components from the methylene chloride extract were identified tentatively. With one exception, guaraná leaves contained, at most, only traces of caffeine.

Rehydration and reduction of the guaraná materials and agitation increased the extraction of caffeine significantly (0.05 level). Greater amounts of caffeine were extracted from alkaline treated seed and ground material.

The following conditions were found to be the most efficient for extracting caffeine: rehydrate the guaraná material with alkaline solution to approximately 40% moisture, use methylene chloride as the solvent, apply constant agitation, and replace the solvent with fresh solvent after specified periods of time.

Coefficients of diffusion for caffeine were determined for six sets of conditions. The were found to be similar to those reported for coffee.

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CHAPTER I

INTRODUCTION

Caffeine is a white, bitter, odorless compound that melts at 236°C. At the present time the two major sources of caffeine are coffee beans, as a by-product of decaffeination, and a synthetic reaction between urea and chloroacetic acid (10). Sivetz and Derosier (36) had earlier reported cocoa waste as the primary commercial source and coffee beans as the secondary source of caffeine; however, caffeine is extracted also from tea leaf waste.

Two-thirds to three-fourths of the quantity of caffeine produced worldwide is used in cola beverages. The remaining caffeine is used in headache, cold, and other remedies (36). Estimates indicate that the United States will consume 45,205.8 million liters of carbonated beverages in 1990 and must import 1,498.2 tons of caffeine to fulfill the requirements of industry (42).

With a population approaching 150 million people, Brazil is presently considered the world's third largest market for soft drink beverages, following that of the United States and Mexico (23). Brazil is the world's largest producer of natural sources of caffeine, producing 45% of the coffee and approximately 30% of the cocoa (26). Brazil is the only country that

cultivates guaraná (*Paulinia cupana* var. *sorbilis* (Mart.) Ducke) (31) on a commercial basis, the richest vegetable source of caffeine known (20). The seed of the guaraná fruit consists of approximately 4.0 to 4.5% caffeine; yet, Brazil remains a traditional importer of caffeine because its caffeine extraction industry is currently in an infant stage.

The objectives of this research were to determine the most appropriate treatments and conditions for extracting caffeine from guaraná seed and to determine the most effective solvents to accomplish the extraction.

CHAPTER II

LITERATURE REVIEW

A. History of Guaraná.

1. Origin of the Plant.

Information on guaraná (*Paulinia cupana* var. *sorbilis* (Mart.) Ducke) (31) was reported first in 1669 by Betendorf, a missionary priest. During his contacts with the Andirás Indians of the Amazon River Basin, he observed that consumption of beverages made from the seeds of this plant was a common habit among tribal members (35). Original studies on the plant were made by Humboldt and Bonpland in 1810. During a trip through the heartland of Venezuela, these explorers collected parts of the plant for botanical classification. The material was described and classified in 1821 as *Paulinia cupana* (33). The first chemical analyses of guaraná were made by Vareg in Cassicourt, England, in 1920. However, the value of guaraná as a beverage ingredient was first recognized by Theodore von Martius who, in the same decade, isolated a chemical component of the plant of which he named "guaranin." Since then, guaraná has been a subject of worldwide interest by the scientific community (30).

Until 30 years ago, guaraná was restricted to Mundurucânia, a region in Brazil located among the Andirá, Maués-Açú, and Paraná dos Ramos Rivers in the southeast part of the State of Amazonas (31). Today, guaraná is cultivated in many parts of Brazil, but the State of Amazonas remains the largest producer, representing 60% of the 1500 tons/year of guaraná produced nationwide. The States of Acre, Rondonia, Mato Grosso, Bahia, and Pará share production of the remaining 40% (28). Brazilian guaraná seed production by state is shown in Figure 1. Maués County in the State of Amazonas is known as the birth place of guaraná (8) and produces 60% of the total crop of the state (11).

2. Taxonomy.

The guaraná plant belongs to the division Angiosperms, class Dicotiledonea, order Spindales, family Sapindaceae, genus Paulinia, section Pleurotoechus, and species cupana (20).

B. Cultural Management Practices.

1. Plant Adaptation.

Guaraná is a low growing plant and its adaptation to field conditions is difficult. It is necessary to select individual plants most resistant to disease and/or with high

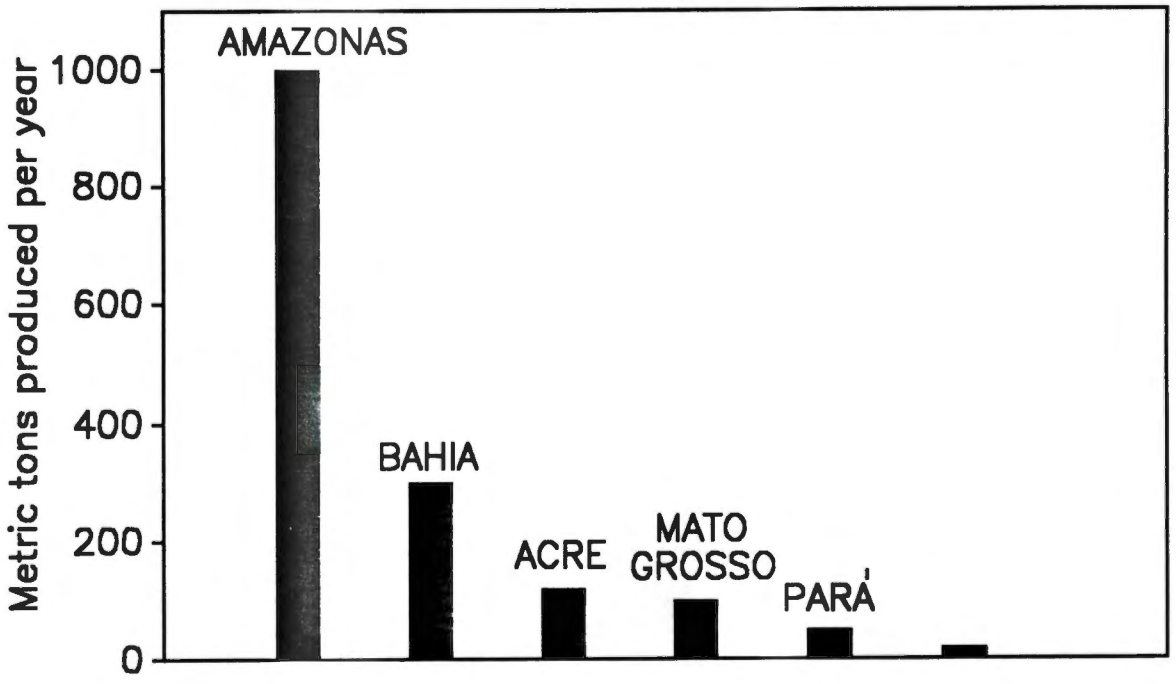

Figure 1. Brazilian production of dried guaraná seed in 1988 by state.

Source: IBGE - Brazilian Statistics Institute, 1986.

productivity to make the crop economically feasible. Another important consideration is the age of the trees when production begins. Traditional guaraná plantations begin production in the 3rd or 4th year, and maximum productivity is obtained usually after the 6th year (20).

2. Propagation.

There are two major methods of propagating guaraná: sexual (seeds) and asexual (vegetative) means. Sexual propagation yields large phenotype variations, even when most appropriate techniques are used. It is common to find highly productive, disease resistant young plants growing side by side with low productive genotypes that have been severely attacked by diseases (8).

Vegetative propagation has proven to be the most efficient method (25). Advantages of using this method include the following points:

- About 38% of the total plant population reaches the mature stage of productivity within 2 years, and about 92% of the plants become fully productive between 3 and 4 years.

- Plants from selected clones produce more uniform and more disease resistant trees. Escobar et al.(22) suggested that cuttings should be obtained from clones free from diseases such as anthracnose and bacterioses, consisting of excellent nutritional appearance, and, if possible, consisting

of a productivity of 1 kg of dry seeds per plant per harvest season.

Presently, at Brazil's experiment station UEPAE near Manaus, propagation is enhanced by the use of indolebutyric acid (an auxin). One kg of the plant growth regulator is sufficient to make 20,000 applications. Procedures and recommendations for use were described by EMBRAPA (20).

3. Nursery Stage.

Guaraná is a very sensitive plant and is allowed to adapt during the first 9 months in nursery houses. These facilities are very rustic, basically made with wooden stakes 2.5 m high and constructed in a square shape 5.5 m^2. The shelter is covered with palm leaves distributed over crossed wooden frames supported by the stakes. For lateral protection, a clear, plastic material of polyvinyl diene chloride, called "Saran," is used to reduce 60% of the sunlight. After the 5th month of growth, the palm leaves are removed gradually to allow increasing amounts of sunlight to fall on the young plants.

The young plants are begun as stem tip cuttings from the mother clone. The cut end of the cutting is dipped in indolebutyric acid and set in a soil medium that is held in polyethylene plastic bags of 1 kg capacity. The bag, soil medium, and its stem cutting are placed into the nursery. The bags are

perforated with approximately 18, 0.5 cm holes uniformly distributed to permit the draining of excess water. Irrigation should provide approximately 600 ml water to each plant each day, an amount sufficient to maintain moisture equilibrium of the plant. The most appropriate soils for guaraná are well-drained, deep soils, with a texture varying from a heavy clay to a sandy clay, and a level terrain. Fertilization is advisable immediately after placing the cuttings in the nursery house. Satisfactory results are obtained with an application of 1.5 g fertilizer containing: 1 g urea, 0.3 g potassium chloride, and 1 g of dipotassium magnesium sulfide. Reapplication is suggested after every 3 months.

A nursery house with sufficient capacity to accommodate the growing of an adequate number of plants for transplanting 1 ha of a plantation should be built close to the source of water and the future plantation. Approximately 650 selected plants are needed to start 1 ha of a new plantation. The minimum size of a house with capacity to produce 1000 plants should consist of an area of 64 m^2. Other suggestions and recommendations about the culture of guaraná plants have been suggested by EMBRAPA (20).

4. Field Adaptation.

After 8 to 9 months in nursery houses, guaraná plants are ready for moving to the field. Careful selection of plants is

recommended to prevent future losses. Ideal plants should be free from diseases, have no physical deformation, and show 9 to 11 leaves with at least 1 having reached the compound formation. Usually, 30% to 40% of the plants are eliminated at this point.

After being transplanted to the field, guaraná plants experience a slow growing process that takes from 2 to 6 years to reach full production, depending upon the propagation method used and cultural and environmental conditions.

5. Production Phase.

During the growing stage, leaves of more than 2 years of age should be removed from the extremities of the terminal branches. Pruning of leaves is recommended only after the first harvest of fruit because the young growth contributes to the growth of the plant. Herbicides may be used when necessary. During the first year, herbicide and urea should be applied to the soil. The herbicide glyphosate (trade name "Round Up") is recommended along with urea in the second and third years. When the plants are more hardy, paraquat (another herbicide) and urea are recommended as a treatment. Procedures and recommendations have been suggested by EMBRAPA (20).

6. Harvesting and Processing the Fruit.

a. Fruit Description. The guaraná fruit has an elongated, capsular form with a pointed distal extremity. At maturity the fruit have an average 2 cm length and 1 cm diameter. The immature fruit is enclosed within a dark, greenish colored shell. At maturity the shell becomes a reddish orange color, the sutures rupture, and the distal end of the seed becomes exposed. The seeds are a dark brown color with a medium to hard crustaceous texture. A fine amylaceous integument covers the seed (20).

b. Harvesting. Harvesting is begun at the end of October and is finished by the following January. A reliable index for harvesting is the development of a condition, whereby, at least 50% of the fruits in the same cluster show a rupture in the distal extremity of the shell. Mature fruit fall easily from the tree, thus manual harvesting is the most convenient method. Pickers remove ripe fruit from the tree and from the ground. Also, guaraná is a peculiar tree with a large inverted bowl shape with many branches, making mechanical harvesting difficult and causing excessive loss of the fruit.

c. Processing of the Fruit. Processing of the fruit may be divided into three phases: fermentation, pulping, and drying.

c1. Fermentation. After being harvested, the fruit are spread to a depth of no more than 25 cm onto a piece of

canvas cloth and allowed to dry by natural means. The fruit is covered for protection from rain while providing adequate ventilation. The drying fruit is held under these conditions for approximately 3 days to allow fermentation to occur and to permit moisture in the fruit to be removed.

c2. Pulping. Removal of the pulp can be accomplished by mechanical or manual means. By following traditional methods, the seeds are separated from the fruit shell after fermentation by allowing individuals to walk on the fruits with the bare feet. A mechanical or manual operation is applied then to remove the residual pulp from the seeds. The seeds without the pulp are then washed on large wire screens with 5 mm openings with flowing water and allowed to dehydrate under sunlight for about 12 hours before the final drying step is begun.

c3. Drying the Seeds. Three types of drying methods are used currently to dry guaraná seeds: traditional, natural, and industrial.

The traditional method is used mostly by small farmers. Seeds are dried on large iron plates that are heated by the burning of wood in a clay oven. The complete process takes 4 to 5 hours under mild temperatures. The locations where this operation takes place are known as "flour houses" because the facilities are used also to process cassava flour.

The natural drying method is simple and efficient. Seeds

are dried directly by the sun's radiation in solar driers. The final moisture level is reduced to 10 to 12% , a level considered to be ideal. The inconvenience of this method is that it requires approximately 4 days for completion, depending upon the weather conditions.

The industrial method is the most sophisticated and the most expensive of the drying methods. An industrial coffee dryer is used to force heated air inside a chamber where the seeds are contacted for approximately 15 minutes. The seeds are dried to a more uniform moisture content by this method than when dried by the two other methods.

C. Uses of Guaraná.

1. Traditional Use.

Similar to coffee, the seed of guaraná is the only part of the plant used for human consumption. The dried seeds are crushed with a large wooden pestle and mortar and mixed with a sufficient amount of water to form a dough. Individual "breads" are prepared and smoked at 40-50°C for approximately 3 days to form a hard, woody textured bar. To consume the bar it is necessary to convert it to a powder. The Indians, as traditional consumers of this product, use the "hioide" bone of the tongue of a fish (Pirarucú, *Arapaima gigas*) of the region to prepare the powder. The bone is used as one might

use sandpaper. The powder produced by the "sanding" action is used to produce beverages (20).

2. Regional Use by Small Companies.

Guaraná seeds are converted into powder and syrup (a mixture of guaraná concentrate and sugar syrup blended with vanilla) by small companies in northern Brazil. The largest of these companies is AMINCO, "Amazônia Industrial e Comercial de Guaraná S.A", located in Manaus, Amazonas. The seeds are purchased from small farmers. The seed coat is removed and discarded, and the seed is milled to pass through a 80 mesh screen. Individual packages of 85 g. guaraná powder are readily available in local retail outlets. Brazilian law requires that guaraná syrup must contain a concentrate prepared with a minimum of 1% dried guaraná seed or an equivalent amount of extracted guaraná concentrate (15, 16, 17). Less than 5% of guaraná seeds produced are used for syrup manufacture.

3. Industrial Use.

About 80% of the guaraná seeds produced in Brazil are converted into a concentrate for use in formulating beverages, mostly soft drinks. The concentrate is produced by a process of slow extraction of soluble substances from the whole seed

by the use of an alcohol-water mixture (60:40, v/v). Soft drinks with guaraná comprise the 2nd most consumed group of beverages in Brazil, capturing 14% of the total market. "Cola" beverages, with 52% of the market, comprise the major group of soft drinks. Brazilian law requires that guaraná soft drinks must contain dried guaraná seed material or an equivalent amount of extract from guaraná concentrate. Maximum and minimum amounts of 0.2% and 0.02%, respectively, have been established (17). Two local companies control 80% of the guaraná soft drink market: Compania Antartica Paulista and Brahma S.A. The Coca-Cola Company and Refrigerantes da Amazônia S.A.-RASA share the remaining 20% of the market (28).

D. Chemical Composition of Guaraná Seeds.

1. Proximate Analysis.

Literature on composition of guaraná seeds is limited. Angelluci et al. (1) analyzed guaraná seeds from Maués County for composition and found that the seed coat represented 18.8% of the weight of the whole seed. The contents of whole seeds were determined to be as follows: moisture (10.47%), protein (13.23%), fat (2.69%), ash (1.44%), fiber (10.23%), and carbohydrate (61.94%). The analysis included the minerals Ca (29 mg%), K (337 mg%), Mg (83 mg%), Na (6 mg%), Cu (0.9 mg%), Mn (2.8 mg%), Zn (1.7 mg%), Fe (2.6 mg%), and $PO_4^{\equiv}$ (344 mg%).

All components except moisture were presented on the dry weight basis.

2. Caffeine Analysis.

Guaraná seed is the richest known source of caffeine. The caffeine concentration in guaraná is 2 to 3 times greater than in the coffee bean, about 2 times greater than in the tea leaves, and about 3 times greater than in the cola nut (36). Literature indicated that caffeine may be found also in other parts of the guaraná plant in addition to the seed. However, values (dry basis) observed for flowers (traces) and leaves (0.38%) are very low when compared to that of the seeds (4.4%) (19).

Variation in the caffeine content of guaraná seeds seems to depend on different factors, including uniformity of samples, maturity at harvest, and the drying method used. Lira (30) reported caffeine concentration of 3.25% to 6.98%, with a mean 3.73%. Angelucci et al.(1) reported a mean concentration for caffeine of 3.28%. Maravalhas (31) found 2.7% to 3.5% caffeine in the seed and 2.27% to 3.00% in the seed coat. Escobar et al. (22) presented mean values of 2.2% to 4.4% in the whole seed. Other researchers reported the caffeine concentration of guaraná seed of: 5.38% (19) and 4.3% to 4.7% (25).

E. Economic Aspects.

1. Production Costs.

Perhaps the most important study about economic aspects of guaraná culture was published by Fundação Centro de Apoio ao Distrito Agropecuário - FUCADA (24), a Brazilian government foundation that provides technical and financial support to small farmers located in the State of Amazonas (24). In this study 100 ha of land were planted in guaraná; 50% of this area was planted in the 1st year (1984), and the remaining 50% was planted in the 2nd year (1985). Cultivation was begun and will continue for 30 years. The first fruits were harvested during the 3rd year after planting. As this project continues and as data become available, FUCADA will report on the feasibility of producing guaraná for its caffeine content.

2. Regional Economic Importance.

Amazonas is the largest Brazilian state, consisting of 540,000 km^2 territory (28). Yet, its participation has been minimal in the national effort to increase Brazilian agricultural exports. Until the present time the contribution of Amazonas has been limited primarily to Brazil nuts (14). However, there are other regional products that might be used to aid in promoting the economic developmental efforts; guaraná is one of these products (11).

In April 1990, The Coca-Cola Company of Brazil will expand its operations by initiating a processing plant in Manaus, Amazonas to produce guaraná concentrate to meet the needs of Brazil and neighboring countries (39). Since guaraná has already been used to produce soft drinks (14% of the Brazilian market) (28) and since the demand of caffeine for making cola beverages consumes two-thirds to three-fourths of the world's total caffeine production (36), efforts have been made to expand the market for guaraná, thereby, improving the economy of the state (24).

3. Use of Caffeine.

Caffeine is a valuable commodity for use as an additive in beverages and pharmaceuticals. At the present time caffeine is used primarily in carbonated beverages and in headache and other remedies (36).

F. Caffeine Extraction Methods.

1. History.

In 1820, Ferdinand Runge, a German chemist, isolated caffeine from coffee beans. This discovery caused considerable excitement because the substance would then become available for use by pharmacies (36).

The process of removing caffeine from a vegetable source

was first developed by Ludwig Roselius and Karl Wimmer of Kaffee-Handels-Aktien-Gesellschaft, Bremen, Germany. A patent letter for this development was issued by DRP Deutsches Reich Patents-chrift in 1905 (10). Caffeine is presently produced from synthetic sources, e.g. methylation of theobromine from cocoa wastes, and is available in an industrial grade of 70%-90% purity and in U.S.P grade of 99.5% purity for use in the manufacturing of medicines (36).

2. Industrial Methods of Decaffeination.

Decaffeination has been a fruitful field for inventions and, therefore, for patents. Despite the large number of patents concerning coffee decaffeination, there are only three different methods in use: organic solvent decaffeination, water decaffeination, and supercritical carbon dioxide decaffeination.

a. Solvent Decaffeination. Use of solvent to extract caffeine from its sources is the methodology preferred by most commercial operations. The most direct approach is to contact the pre-wetted material with an appropriate solvent in conventional equipment in either the batch or continuous systems. According to Katz (29), the only known commercial solvents used on the commercial scale other than methylene chloride are ethyl acetate, coffee oil and other triglycerides.

Sivetz and Derosier (36) divided the method of extraction into five steps as follows: 1 the moisture content of the material (green coffee) should be increased from 10% to about 40% by steaming; 2 the material should be extracted with an organic solvent by countercurrent flow for 12 to 18 hours, time sufficient to extract 97% of the caffeine; 3 the solvent fraction containing the caffeine should be collected and concentrated by steam distillation; 4 the material should be heated with live steam to remove the residual solvent, and 5 the caffeine sludge obtained from the steam distillation should be refined and dried.

b. Water Decaffeination. The water decaffeination process was developed in 1941 by Berry and Walters of the General Foods Corporation to eliminate the use of organic solvents and, consequently, prevent the presence of solvent residues in green coffee (29). With this methodology, materials are extracted by countercurrent flow in a battery of percolation columns using hot water. After extraction has been finished, the extracting water is cooled and the caffeine crystals are formed and removed through application of a liquid/liquid (GATX) extraction technique, using methylchloroform (TCE), a chlorinated solvent. The caffeine is recovered from TCE by steam distillation, refined, and dried.

c. Supercritical Carbon Dioxide Decaffeination. Extraction of caffeine by use of supercritical carbon dioxide was discovered by Kurt Zosel at the Max Planck Institute für Kohlenforschung at Mülheim-Ruhr, Germany, in 1959 and is being investigated presently at several institutes over the world (18). Use of carbon dioxide for decaffeination of green coffee beans is similar to that of other solvents, except that this solvent is inert. Under appropriate conditions of pressure (72.9 bar), volume (468 g/l), and temperature (31°C) the gas becomes liquefied in an enclosed system in which the caffeine-containing material is placed. The caffeine is extracted from the source and then removed from the solvent. The liquid carbon dioxide is recirculated to continue the extraction process. According to Martin (31), the use of critical carbon dioxide is in most cases more selective, non-polluting, non-toxic, and more effective than the low boiling organic solvents. According to Katz (29) the major disadvantages of using such pressures are the requirement of costly equipment and batch processing.

3. Refining and Drying.

Refining is a process that combines a number of purifying steps to eliminate various contaminants such as waxes, oils, and caffeine-like water soluble materials that impart a dark color to a coarse granular crystal instead of the desired

white, needle-shaped crystals of pure caffeine (10).

The unrefined caffeine derived from the decaffeination process is usually obtained in a aqueous solution or as a dried cake. Any residual solvent present is steam-distilled and the caffeine sludge that is formed is redissolved with recycled mother liquid from a centrifugation step in the refining process. Activated carbon is added to remove most color-contributing impurities. The slurry is filtered and the spent carbon is discarded or reactivated and reused. The caffeine present in the liquid filtrate may be recovered by extraction with acetic acid and by further solvent separation by steam distillation, or by centrifuging the crystals and by reducing the moisture of the cake from 35% to 0.25%. The drying operation is carried out under partial vacuum in a specialized unit called a Turbo-Dryer. At this stage the caffeine is 80 to 90% pure (10).

During World War II, Brazil suspended its caffeine shipments from Germany and developed a technique to extract caffeine from tea leaves. The process consisted of 5 steps:

a. Alkalinization. The leaves were contacted with a solution of 10% calcium oxide (CaO) for a period necessary to rehydrate the material.

b. Extraction. The caffeine was extracted by counter-current flow with a petroleum derivative such as kerosene.

c. Solvent Recovery. After completion of the extraction

process, the solvent was recovered from the mixture by steam distillation and the slurry consisting of water, caffeine, and extraneous materials was collected.

d. Clarification and Crystallization. The slurry was suspended in hot water and fine activated carbon was poured into the suspension. After allowing the activated carbon to trap the contaminants, the suspension was filtered under pressure, and the liquid filtrate was cooled and centrifuged to yield a mass containing 70% caffeine.

e. Recrystalization. The mass obtained from the crystallization step was resuspended in hot water and step "d" was repeated. The newly centrifuged residue, containing 75% caffeine, was dried in a conventional oven.

This method was used with success on seed coat material of guaraná (31). An operational flow sheet was presented.

CHAPTER III

MATERIALS AND METHODS

A. Source of Guaraná Seeds and Leaves.

Dried seeds of guaraná (*Paulinia cupana* var. *sorbilis* (Mart.) Ducke) were acquired in the State of Amazonas, Brazil. Thirteen kg of traditionally dried seeds were obtained from AMINCO, a privately owned guaraná plant located in Manaus, and 3 kg of naturally dried seeds were obtained from UEPAE-MAUÉS, a government experimental station near Maués, Amazonas. Traditionally dried seeds were dried on large iron plates that were heated by the burning of wood in a clay oven, and naturally dried seeds were dried directly by the sun's radiation in solar driers.

The leaves of 17 clones were collected at UEPAE-MANAUS near Manaus, Amazonas. All leaves from each clone were 2 years old and were collected at 2 different times: 2 months before the harvesting of fruit was begun and 2 months after all the fruit had been harvested. Individual clones from which the leaves were collected varied from 3 to 6 years of age. Immediately after being collected, the leaves were dried at 100°C in a conventional oven with circulating air and then ground into a powder.

The seed and leaf materials were packaged in plastic bottles and brought to the University of Tennessee by a faculty member. Permission to import the materials was granted by The Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Hyattsville, MD. The seed and leaf materials were held in plastic containers at -17°C in the Food Technology and Science laboratories until used for analyses.

B. Methods of Chemical Analyses.

1. Proximate Analysis.

Samples of traditionally- and naturally dried guaraná seeds were analyzed for the components presented below. Three replications of each type of seed were analyzed for all components except for dietary fiber for which only two replications were analyzed.

a. Moisture. Moisture content of guaraná seeds was determined by the AOAC Vacuum Oven Method (2). Samples of 2 grams were dried to constant weight at 70 °C overnight at a partial vacuum of 163 torr. Percentage of moisture was calculated.

b. Crude Protein. Total nitrogen was determined by the AOAC micro-Kjeldahl method (2). One hundred milligram samples were analyzed for total nitrogen content. Percentage of crude protein was calculated (% N x 6.25) on the dry weight basis.

c. Crude Fat. Samples of oven-dried material were extracted for 16 hours with petroleum ether in a Goldfish Apparatus according to the AOAC (2) procedure. Percentage of crude fat was calculated on the dry weight basis.

d. Ash. The percentage of ash was determined according to the AOAC (2) procedure. Two grams of samples were ashed in a muffle furnace at 525°C until the sample reached constant weight. Percentage of ash was calculated on the dry weight basis.

e. Dietary Fiber. Total dietary fiber was determined by an enzymatic-gravimetric method (4) using the Fibertec System, model E (Tecator Co., Hoganas, Sweden).

f. Carbohydrates. Percentage of carbohydrate was calculated by subtracting the percentages of crude protein, crude fat, ash, and dietary fiber from 100%.

2. Minerals.

The contents of calcium, magnesium, potassium, copper, manganese, iron, zinc, sodium, and phosphorus were determined on traditionally- and naturally dried guaraná seeds, using an Instrumentation Laboratories Atomic Absorption-Emission Spectrophotometer (model 551 AA/AE) (21). Replications of 2 gram samples of the ground seed material were ashed at 550°C, dissolved in HCl of 6 N solution, and diluted according to the mineral analyzed. The mineral content was determined and

reported as milligrams/100 gram material (dry basis).

3. Alkaloids.

The alkaloid contents of the dry seeds and leaves of guaraná were determined, using a modification of a method developed by Ashor et al. (3). The alkaloids were extracted and analyzed by the following procedure. Approximately 30 g of the material were dried to constant weight at 70°C overnight at a partial vacuum of 163 torr. Three samples of 0.5 g each were weighted and placed into a 250 ml beaker. Approximately 100 ml of distilled water were added to the sample, and the mixture was heated with stirring at 90°C for 20 minutes. After cooling to ambient room temperature, the mixture was filtered on Whatman No. 1 paper, and the filtrate was adjusted to pH 10 with 2.5% NaOH solution. The filtrate was extracted twice with two times the volume of chloroform ($CHCl_3$) and passed through anhydrous Na_2SO_4. The initial volume was reduced to approximately 10 ml in a rotatory evaporator and then evaporated to dryness under nitrogen gas. Approximately 50 ml of boiling water were added to the residue, and the mixture was transferred to a 250 ml flask. This operation was repeated three times. After cooling to room temperature, the mixture was brought to volume of 250 ml and filtered on Whatman No. 1 paper. An aliquot of 5 ml was diluted with High Pressure Liquid Chromatograph (HPLC) water and brought to volume in a

100 ml volumetric flask. An aliquot of 20 ml of the solution was filtered on a 0.45 μm membrane filter (millipore), and volumes of 10 to 20 μl were injected into a HPLC instrument for analysis. A Waters HPLC Instrument (Waters Assoc., Milford, MA, model 6000A solvent delivery system with a U6K injector) was used and the alkaloids were separated with a Waters μ-Bondapak C-18 millipore column. The alkaloids were detected by a Waters Lambda Max (model 440) Spectrophotometer Absorbance detector at 280 nm. The mobile phase used in the system was a solution of water-methanol (60:40, v/v) with 1% acetic acid. A 1.0 ml per minute flow rate was used during the determination.

The detector response was correlated to the alkaloid concentration by the use of a Hewlett Packard (Model HP-3393A) computing integrator. A standard solution of alkaloids containing 10 μg/ml theobromine, 10 μg/ml theophylline, and 15 μg/ml caffeine was prepared. From the solution, 8 different volumes varying between 10 and 25 μl were used to develop a standard curve. Results were expressed as g% of caffeine.

4. Flavoring Substances.

a. Extraction. The guaraná samples collected for analysis of volatile compounds were stored in sealed polyethylene bags at -17°C until analyzed. The volatiles were isolated from a sample of 150 g of ground seed material in the following

manner. The seeds were ground with a Viking Hammer Mill to pass through screen openings of 1.5 mm. The ground material was blended for 5 minutes in an Osterizer blender at high speed with 700 ml HPLC grade water of 60-70°C. The aqueous guaraná slurry was poured into a round bottom flask of 1000 ml capacity that was equipped with a side tube for nitrogen delivery to the bottom of the flask. The flask was connected to a simultaneous distillation extraction (SDE) apparatus. Nitrogen was bubbled through the slurry to prevent oxidation of the sample during extraction. The flask with the slurry was heated in an oil bath at 125-130°C and a 125 ml Erlenmeyer flask containing 45 ml of HPLC grade methylene chloride was connected to the steam distillation equipment (SDE) for extraction of the volatiles (5). The volatiles were extracted for 2 hours and cooled to room temperature; the methylene chloride extract that contained the volatiles was collected. At this point, 1 ml of methanol that contained 0.1 mg of pyrogallol was added to the volatile extract to prevent oxidation. The extract was concentrated to approximately 1 ml according to the procedure recommended by Au-Yeung and MacLeod (5). All glassware used in this analysis was cleaned according to the following procedure:

- Washed with the detergent "Alconox" and warm water
- Rinsed with deionized water
- Rinsed with acetone

- Rinsed with HPLC grade water
- Rinsed with 4% glacial acetic acid
- Rinsed with deionized water
- Rinsed HPLC grade water

b. Qualitative Analysis. Volatiles in each sample were analyzed qualitatively on a Shimadzu Model 9AM gas chromatograph equipped with a 30 m by 0.25 mm i.d. fused silica SP2330 capillary column (Supelco, Inc.) interfaced directly with a Shimadzu QP 1000 mass spectrometer (GCMS analysis).

A tentative identification of a volatile was made by matching its mass spectrum with that of a known compound via the computer search of a library mass spectrum. A blank using 700 ml HPLC grade water was run to determine the presence of artifacts.

The conditions for the GCMS analysis of guaraná seed volatiles were as the following:

The injector temperature was adjusted to 250°C. The initial and the final holding times were 2.5 and 30 minutes, respectively. The initial and the final temperatures of the column were 25 and 220°C., respectively. The column temperature was increased at the rate of 2°C per minute. The gas carrier was helium and the ion source was heated to 250°C with an energy of 70 eV.

C. Physical Measurements.

1. Seed Dimension.

The length and diameter of guaraná seeds were measured with a caliper on 50 randomly selected seeds. The longitudinal and latitudinal diameters were used to calculate the mean radius according to the following equation:

$$r_o = \frac{d_1 + d_2}{4} \qquad (1)$$

Where:

d_1 = longitudinal mean diameter in cm.

d_2 = latitudinal mean diameter in cm.

r_o = radius in cm.

The radius was used in the calculation of diffusion coefficients as described in Section G.

2. Color.

The color of ground, dry guaraná seed material was measured on traditionally- and naturally dried samples. A Hunter Color Meter (model D25D2M) was used. The meter was standardized with a white tile (Hunter C-2-21125; L = 91.03, "a" = -1.3, "b" = 1.6). The samples from each treatment were placed into a cuvette constructed with an optical glass bottom, and the L, "a", and "b" values were measured and recorded.

3. Firmness.

Firmness was determined on whole, dry guaraná seed by using an Instron Food Testing Machine (model 1132). A 500 kg capacity load cell was used with a crosshead speed of 10 cm per minute. A Kramer Shear-Compression Cell was utilized to test samples of 10 guaraná seeds. Firmness, the force required to shear the sample, was expressed as Force (H) and Force (A). Force (H) was equal to the peak height of the work-force curve divided by the weight of the sample, and Force (A) was equal to the area under the work-force curve divided by the weight of the sample.

D. Preparation of Seed for Caffeine Extraction.

Traditionally dried seeds were dried to constant weight at 70°C overnight at a partial vacuum of 163 torr. Preparation of seeds for extraction of caffeine was made as follows:

1. Whole Seeds.

a. Seeds received no preparation before extraction of caffeine. Extraction was accomplished by contacting the seeds with a solvent of a low boiling temperature for designated periods of time.

b. Seeds were rehydrated in HCl solution of 0.1 M to allow the moisture content to increase to 40% and steamed for

30 minutes.

c. Seeds were rehydrated in NaOH solution of 0.1 M to allow the moisture content to increase to 40% and steamed for 30 minutes.

2. Ground Seeds.

a. Seeds were ground in a conventional Viking hammer mill (model T-102), equipped with a screen of 1.50 mm (diameter) openings. The particle size distribution was obtained by passing 500 g of the ground seed material through a set of seven sieves stacked one over the other. The sieves were arranged in decreasing order of screen openings. The stack of sieves was held in a shaker device that was operated by a 1/2 h.p. motor. The mesh numbers and opening sizes (mm, diameter) of the sieves were the following: 10 (2.00), 20 (1.38), 30 (0.59), 50 (0.30), 60 (0.25), 70 (0.20), and 100 (0.15). The distribution of the particle sizes was reported in percentage of the total amount of material.

b. Ground material (Section 2. a.) was rehydrated in HCl solution of 0.1 M to allow the moisture content to increase to 40% and steamed for 30 minutes.

c. Ground material (Section 2. a.) was rehydrated in NaOH solution of 0.1 M to allow the moisture content to increase to 40% and steamed for 30 minutes.

E. Extraction Procedures.

Extraction of caffeine from all seed materials as described in Section D was conducted with one or more of the following solvents: 40% ethanol-60% water, 60% ethanol-40% water, 95% ethanol-5% water, methylene chloride, and ethyl acetate. The extraction processes used and the conditions applied were as follows:

1. Single-stage Process.

Material was contacted with the solvent for specific periods of time, as in a batch system. The solvent and extracted material were recovered when equilibrium had been approached. Yields (%) of caffeine in the extract were calculated by subtracting the value of the amount of caffeine that remained in the extracted material from the value of the amount of caffeine that was present in the seed prior to extraction.

2. Simple Multiple-stage Process.

This process is similar to that in Section E. 1. but with the following exceptions: after an extraction period of 6 hours, the solvent was discarded and a sample of 5 seeds was withdraw from the extraction vessel for caffeine measurement. Fresh solvent was added to the flask and the extraction

process was allowed to run an additional 6 hours. These procedures were continued for a total extraction period of 30 hours (40). Yields (%) of caffeine extracted were calculated according to the procedure described in Section E. 1.

3. No Agitation Condition.

Three replications of 100 g samples of seeds or ground seed material were extracted, using the single-stage process for holding the seeds in contact with a solvent for 4-hour periods, ranging from 0 to 28 hours, in tightly sealed 500 ml Erlenmeyer flasks at approximately 25°C. Not all treatments were extracted for 28 hours, however. Maximum periods for extraction are presented in Chapter IV. The seed/solvent ratio used on the extractions was 1:4 (w/v). The mixture of seed and solvent was allowed to set without agitation or application of other actions. Samples of the extracted material were collected at the specified times, filtered through Whatman No. 1 paper on a Buchner funnel with vacuum, dried under partial vacuum, cooled in a desiccator, and analyzed for caffeine content according to the procedure of Ashor et al. (3)

4. Constant Agitation Condition.

The procedure for extraction with the single-stage or multiple-stage process with constant agitation was similar to that with no agitation except that the flasks with the mixture

of seed and solvent materials was agitated constantly on a Burrel wrist action shaker, Model-75. The number of agitations was 4 per second and the yields (%) of caffeine extracted were calculated according to the procedure described in Section E.

F. Treatments for Extracting Caffeine.

In order to compare extraction properties of the 5 solvents (Section E) on the six preparations (Section D), the following treatments (TMT) were applied to traditionally dried seeds:

TMT 1 - Whole seeds without further preparation (Section D. 1. a) were extracted with the single-stage process under no agitation for 20 hours (Section E. 1). The three solvents tested were 40% ethanol-60% water, 60% ethanol-40% water, and methylene chloride.

TMT 2 - Whole seeds without further preparation were extracted with the single-stage process under constant agitation for 24 hours (Section E. 2). The two solvents used were 60% ethanol-40% water and methylene chloride.

TMT 3 - Whole seeds that had been rehydrated in HCl solution of 0.1 M or NaOH solution of 0.1 M to allow the moisture content to increase to 40% (Sections D. 1. b and D. 1. c) and steamed for 30 minutes were used. The seeds were extracted with the single-stage process under constant agita-

tion for 26 hours (Section E. 2). The solvent used was methylene chloride.

TMT 4 - Ground seed material without further preparation (Section D. 2. a) was extracted with the single-stage process under no agitation or with the single-stage process under constant agitation for 28 hours (Section E. 1 and Section E. 2). The solvent used was methylene chloride.

TMT 5 - Ground seed material that had been rehydrated in NaOH solution of 0.1 M to allow the moisture content to increase to 40% and steamed for 30 minutes (Section D. 2. c) was extracted with the single-stage process under constant agitation (Section E. 2) or with the multiple-stage process under constant agitation (Section E. 3) for 30 hours. The solvent used was methylene chloride.

TMT 6 - Ground seed material that had been rehydrated in NaOH solution of 0.1 M to allow the moisture content to increase to 40% and steamed for 30 minutes (Section D. 2. c) was extracted with the multiple-stage process with constant agitation for 30 hours (Section E. 3). The three solvents tested were 95% ethanol-5% water, methylene chloride, and ethyl acetate.

G. Extractions Used for Calculation of Diffusion Coefficients of Caffeine.

Data from six experiments as described in Section E were used to calculate diffusion coefficients for the extraction of caffeine from whole seed and ground seed material (Sections D. 1 and D. 2) The experiments conducted were the following:

1. Whole Seeds.

a. Seeds without further preparation were extracted with 60% ethanol-40% water (v/v) for 24 hours, using the single-stage process with no agitation or with constant agitation as described in Sections E. 1 and E. 2, respectively.

b. Seeds without further preparation were extracted with methylene chloride for 24 hours, using the single-stage process with no agitation or with constant agitation as described in Sections E. 1 and E. 2, respectively.

c. Seeds that had been previously rehydrated in HCl or NaOH solutions of 0.1 M to allow the moisture content to increase to 40% were extracted with methylene chloride for 26 hours, using the single-stage process with constant agitation as described in Section E. 2.

Since extraction was allowed to occur over a long period of time (>20 hours), the following equation was used to calculate the diffusion coefficients for whole seeds (13, 41).

$$\ln \left[\frac{\pi^2}{6} \left(\frac{C - C_f}{C_i - C_f} \right) \right] = \left(-\frac{1}{\tau}\right) t \qquad (2)$$

Where:

$$\tau = \frac{r_o^2}{\pi^2 D} \qquad (2a)$$

t = contacting time, seconds.

C_i = initial caffeine content of seeds, % (dry basis).

C = caffeine content of seeds at time, t.

C_f = final caffeine content of seeds, % (dry basis)

D = diffusion coefficient, cm^2/sec.

r_o = radius of seeds, cm (Section C. 1).

2. Ground Seed Material.

a. Ground seed material without further preparation was extracted with methylene chloride for 28 hours, using the single-stage with no agitation or with constant agitation as described in Sections E. 1 and E. 2, respectively.

b. Ground seed material that had been previously rehydrated in NaOH solution of 0.1 M to allow the moisture content to increase to 40% was extracted with methylene chloride for 30 hours, using the single-stage process with

constant agitation or the multiple-stage process with constant agitation as described in Sections E. 2 and E. 3.

c. Ground seed material that had been previously rehydrated in NaOH solution of 0.1 M to allow the moisture content to increase to 40% was extracted with 95% ethanol-5% water, methylene chloride, or ethyl acetate for 30 hours, using the multiple-stage, batch process with constant agitation as described in Section E. 3.

To determine the apparent diffusion coefficient (D') for the ground seed material, Equation 2 was modified to take into account the fact that ground material of smaller particle sizes would be used. (D') was expressed as $sec.^{-1}$ as given in Equation 3. The derivation of equation 3. is presented in appendix A.

$$\ln \left[\frac{\pi^2}{6} \left(\frac{C - C_f}{C_i - C_f} \right) \right] = - D' t \qquad (3)$$

The left hand side of Equations 2 and 3 when plotted against time, t, yields a straight line with the slope, $-1/\tau$ and $-D'$, respectively . For each treatment, the slope of the resulting curve was obtained through linear regression analysis, and the diffusion coefficient, D, and the apparent diffusion coefficient, D', were calculated.

H. Experimental Procedure and Statistical Analyses.

1. Physical and Chemical Analyses of Seeds.

In order to compare the composition of seeds from the two drying methods, three samples were obtained from each group at random and analyzed for each component except for mineral content and dietary fiber for which only two samples were analyzed. The data for proximate components, minerals, caffeine, color, and firmness were compared with F-tests. Significant differences between means were reported at 0.01 and 0.05 levels (37).

2. Extraction of Caffeine from Seeds.

Samples were prepared and analyzed in terms of the percentage of caffeine extracted. This was accomplished under varying conditions, and each set of conditions was considered a treatment (Section F). Each treatment was analyzed by analysis of variance as a factorial of a completely randomized design. The following factors were considered for the individual treatments:

TMT 1. Solvents X time (3 X 5).

TMT 2. Solvents X time (2 X 6).

TMT 3. Rehydration solutions X time (2 X 5).

TMT 4. Agitation X time (2 X 7).

TMT 5. Extraction type X time (2 X 5).

TMT 6. Solvents X time (3 X 5).

Student-Newman-Keul's multiple range test was used to determine significance among the means at 0.05 and 0.01 probability levels (38)

An exponential model was used to fit a curve for the data (% caffeine extracted) for each solvent within the treatments. Significance among the curves was determined with the use of F-tests.

3. Determination of Alkaloids in Leaves.

The contents of alkaloids of guaraná leaf material was analyzed, using analysis of variance as a factorial: harvest X clone (2 X 17) . Significance among the means was determined according to Steel and Torrie (37).

All the data were analyzed using Statistical Analysis System (34), and computing was conducted at The University of Tennessee Computing Center.

CHAPTER IV

RESULTS AND DISCUSSION

A. Proximate Composition, Mineral Composition, and Caffeine Content of Guaraná Seeds.

1. Proximate Composition.

The sums of squares from the analysis of variance of the proximate composition of traditionally- (TD) and naturally dried (SD) guaraná seeds are shown in Table I. The moisture content of the seeds was affected by the drying method (0.01 level). SD seeds were exposed to sunlight for approximately 4 days, while the TD seeds were heated directly in large iron bowls with burning wood. The level of all the other components was affected significantly (0.01 or 0.05 levels) by either the drying methods or by use of seeds of different sources (Chapter III, A) as the seeds were collected from different locations. The moisture, and dietary fiber of TD seeds were significantly higher at the 0.01 level, and the protein, fat and ash were significantly higher at the 0.05 level. Carbohydrate content of TD was significantly lower (0.01 level).

The mean values for the components of dried seeds as affected by drying methods or by the source of the seeds are presented in Table II. The values for components of the TD

Table I. Sums of squares from the analysis of variance of proximate components of traditionally- and naturally dried guaraná seeds.

Source of Variation	df	Component	Sum of Squares
		Moisture	
Drying method	1		7.260**
Error	4		0.046
		Crude Protein	
Drying method	1		6.000*
Error	4		1.765
		Crude Fat	
Drying method	1		0.094*
Error	4		0.021
		Ash	
Drying method	1		0.073*
Error	4		0.031
		Dietary Fiber	
Drying method	1		47.596**
Error	2		0.160
		Carbohydrate	
Drying method	1		67.646**
Error	2		0.549

* Significant at 0.05 level.
** Significant at 0.01 level.

Table II. Mean[1] proximate composition of traditionally- and naturally dried guaraná seeds.

Seed Type	Moisture	Proximate Composition of Solids, % (dry basis)				
		Crude Protein	Crude Fat	Ash	TDF[2]	Carbohydrate
Traditionally dried	8.70^{x}	16.12^{a}	2.20^{a}	1.83^{a}	41.19^{x}	38.65^{y}
Naturally dried	6.50^{y}	14.12^{b}	1.95^{b}	1.61^{b}	34.30^{y}	48.02^{x}

[1] Mean of 3 observations with exceptions of 2 observations for dietary fiber and carbohydrate.
[2] Total dietary fiber.
[ab] Means within a factor are significantly different at 0.05 level.
[xy] Means within a factor are significantly different at 0.01 level.

seeds agreed closely with published values(1, 7, 12). After the caffeine has been extracted, the spent material may be a fair source of protein and an excellent source of dietary fiber and carbohydrates. Thus, the material might have use in animal feed or as a source of dietary fiber for human foods.

2. Minerals.

The sums of squares from the analysis of variance of mineral components of TD and SD guaraná seeds are shown in Table III. Of the 9 minerals analyzed, calcium, potassium, copper, and iron were significantly affected.

The mean values for the minerals present in the seeds are shown in Table IV. TD seeds contained more calcium, potassium and copper (0.05 level), and about three times the amount of iron (0.01 level) than the naturally dried seeds. During the traditional drying operation, the seeds were in direct contact with the iron metal of the heating/drying vessels. This process probably contributed to the higher quantities of iron in the seeds. The differences for calcium, potassium, and copper contents were most likely due to natural differences between the two sources of seeds. Variation in soyl types, as well as, in the fertilizers used on the plantations may have contributed to the differences.

Table III. Sums of squares from the analysis of variance of mineral components of traditionally- and naturally dried guaraná seeds.

Source of Variation	df	Component	Sum of Squares
		Calcium	
Drying method	1		7.480*
Error	2		0.498
		Magnesium	
Drying method	1		165.508ns
Error	2		67.678
		Potassium	
Drying method	1		3779.176*
Error	2		122.923
		Copper	
Drying method	1		1.134*
Error	2		0.043
		Manganese	
Drying method	1		0.160ns
Error	2		0.250
		Iron	
Drying method	1		73.874**
Error	2		0.290

Table III. (continued)

Source of Variation	df	Component	Sum of Squares
		Zinc	
Drying method	1		0.062^{ns}
Error	2		0.151
		Sodium	
Drying method	1		1.071^{ns}
Error	2		2.385
		Phosphorus	
Drying method	1		0.308^{ns}
Error	2		19.552

* Significant at 0.05 level.
** Significant at 0.01 level.
ns Not significant at 0.05 level.

Table IV. Mean[1] mineral composition of traditionally- and naturally dried guaraná seeds.

	Mineral Composition of Solids, mg/100 g (dry basis)								
Seed Type	Ca	Mg	K	Cu	Mn	Fe	Zn	Na	P
Traditionally dried	14.67[a]	124.32[a]	555.20[a]	3.97[a]	2.05[a]	14.66[x]	2.80[a]	6.90[a]	17.49[a]
Naturally dried	11.93[b]	111.46[a]	523.98[b]	2.90[b]	1.65[a]	6.06[y]	3.05[a]	5.86[a]	16.94[a]

[1] Mean of 2 observations.
[ab] Means within a factor are significantly different at 0.05 level.
[xy] Means within a factor are significantly different at 0.01 level.

Caffeine Content of Seeds.

The sum of squares from the analysis of variance of the caffeine contents of TD and SD dried guaraná seeds are shown in Table V. No significant difference (0.05 level) was found between the caffeine contents of seeds from the two drying techniques. The overall mean values of 4.40% and 4.07% that were found for TD and SD dried seeds, respectively, were in close agreement with values published by Angelucci et al. (1), BRAHMA (7), Escobar et al. (22), and Nazaré and Figueiredo (33). Since the seeds were obtained from different sources, it is apparent that source had litte effect on caffeine content.

B. Flavoring Volatiles in Guaraná Seeds.

Results of a tentative identification of volatiles present in TD guaraná seeds are shown in Table VI. Fifteen components were identified tentatively by matching the mass spectrum of each component with spectra of known compounds via computer search of a library of mass spectra. Probability of correct identification ranged from 78% for decadienal to 22% for dimethyl heptane, isoamylbenzyr ether, and musk xylol. The volatile components included a diverse group of chemical substances. Further research should be conducted to identify more completely the volatile components of guaraná seed under other conditions of extraction and methods of identification.

Table V. Sum of squares from the analysis of variance of caffeine contents of traditionally- and naturally dried guaraná seeds.

Source of Variation	df	Component	Sum of Squares
		Caffeine	
Drying method	1		0.163[ns]
Error	2		0.127

[ns] Not significant at 0.05 level.

Table VI. Volatiles of a methylene chloride extract from traditionally dried guaraná seeds as determined by mass spectrometry and identified tentatively by matching the mass spectrum of each component with spectra of known compounds via a computer search of a library of mass spectra.

Retention Time (minutes)	Component as Identified	Matching Probability (%)
10.1	1-octen-3-ol	52
21.2	2,5 dymethylpyrazine	52
24.2	carophyllene	45
27.1	pyrrole	39
30.2	nerolidol	47
33.1	allyl benzoate	32
36.1	nerol	39
38.3	hexadecanal	58
41.8	acetophenone	63
42.0	dimethyl heptane	22
42.7	isoamylbenzyl ether	22
50.9	decadienal	78
51.6	musk xylol	22
59.0	ethyl caproate	25
78.0	diethyl phytalate	53

Some, if not all, of these volatiles may contribute to the flavor of finished products that contain guaraná. However, since guaraná seed extract is present in small amounts in soft drink beverages, varying from 0.2% to 0.02%, the flavor components of guaraná may have little or no effect on flavor of the beverages. The major reason for using guaraná extract is to provide caffeine, a stimulant. The use of imitation or natural flavoring agents is common among the producers of guaraná soft drink in Brazil. On the other hand, in products such as syrups or concentrates in which guaraná is present in larger concentrations, the flavor of guaraná may contribute to the overall flavor of these products.

C. Color and Firmness Measurements.

1. Color

The sums of squares from the analysis of variance of Hunter color measurements of TD and SD dried guaraná seeds are presented in Table VII. Luminosity (L), redness ("a"), and yellowness ("b") were affected by the drying methods (0.01 level). The different temperatures used with the two methods are most likely responsible for the color difference. The SD seeds were dried by the sun, while the TD were dried by heating to higher temperatures.

Table VII. Sums of squares from the analysis of variance of Hunter color measurements of traditionally- and naturally dried guaraná seeds.

		Sum of Squares		
Source	df	L	"a"	"b"
Drying method	1	79.94**	10.94**	6.00**
Error	4	0.25	0.05	0.04

** Significant at 0.01 level.

The mean values for the color measurements as affected by the drying methods are presented in Table VIII. TD dried seeds had the higher luminosity (L), redness ("a"), and yellowness ("b"). These differences in color were probably due to chemical reactions (caramelization?) that resulted from the seeds being dried at different temperatures.

2. Firmness.

The sums of squares from the analysis of variance of firmness are shown in Table IX. No significant difference in firmness was found between samples of seeds from the two drying techniques. The firmness values may indicate the degree of difficulty that one might encounter in grinding the seeds. Since there was no difference in firmness of the seeds of the two groups, one may assume that all the seeds could be ground by the same procedure.

Table VIII. Mean[1] values for Hunter color measurements of traditionally- and naturally dried guaraná seeds.

	Color Measurement		
Seed Type	L	"a"	"b"
Traditionally dried	44.87[x]	10.27[x]	16.60[x]
Naturally dried	37.57[y]	7.57[y]	14.60[y]

[1] Mean of 3 observations.
[xy] Means within a factor are significantly different at 0.01 level.

Table IX. Sums of squares from the analysis of variance of firmness of traditionally- and naturally dried guaraná seeds.

		Sum of Squares	
Source	df	Force H[1]	Force A[2]
Drying method	1	288.78[ns]	8975.66[ns]
Error	4	196.28	1300.46

[1] Peak height/sample weight.
[2] Area/sample weight.
[ns] Not significant at 0.05 level.

D. Extraction of Caffeine from Guaraná Seeds

1. Whole Seeds.

a. Treatment 1 - The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted from unprocessed seeds with the single-stage, no agitation method are presented in Table X. The factors, time, solvent, and interaction had significant (0.01 level) effects on the percentage of caffeine extracted.

The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted with the three solvents within each of the time periods and extracted at the five time periods within each of the solvents are presented in Tables XI and XII, respectively. Both factors, solvent within an individual time and time within individual solvent, had significant (0.01 level) effects on the percentage of caffeine extracted.

The mean values of the amounts of caffeine (%) extracted from the seeds of Treatment 1 are presented in Table XIII. For each solvent the amount of caffeine extracted was increased with time, and for this reason, discussion of extraction levels at the shorter periods of time seems to be of little value and will not be presented. The amounts of caffeine extracted with 60% ethanol-40% water were greater than the amounts extracted with the two other solvents at 16 and 20

Table X. Sums of squares from the analysis of variance of caffeine (%) extracted from whole guaraná seeds with three solvents[1], no agitation, and over a 20-hour period[2] as described in Treatment 1[3].

Source	df	Sum of Squares
Time (T)	4	1526.70**
Solvent (S)	2	1985.82**
T X S	8	324.94**
Error	18	17.90

[1] Solvents: 60% ethanol-40% water, 40% ethanol-60% water, and methylene chloride.
[2] Time periods: 4, 8, 12, 16, and 20 hours.
[3] Chapter III, F.
** Significant at 0.01 level.

Table XI. Sums of squares from the analysis of variance of caffeine (%) extracted with the three solvents[1] within each of the time periods of Treatment 1[2].

Source	df	Sum of Squares
Solvents within 4 hours	2	15.94**
Error	5	7.92
Solvents within 8 hours	2	615.82**
Error	5	3.67
Solvents within 12 hours	2	532.11**
Error	5	1.35
Solvents within 16 hours	2	523.29**
Error	5	0.26
Solvents within 20 hours	2	641.60**
Error	5	4.69

[1] Solvents: 60% ethanol-40% water, 40% ethanol-60% water, and methylene chloride.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XII. Sums of squares from the analysis of variance of caffeine (%) extracted at the five time periods[1] within each of the solvents of Treatment 1[2].

Source	df	Sum of Squares
Times within 40% Ethanol-60% Water	4	232.59**
Error	10	4.69
Times within 60% Ethanol-40% Water	4	615.82**
Error	5	3.67
Times within Methylene Chloride	4	532.11**
Error	10	1.35

[1] Time periods: 4, 8, 12, 16, and 20 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XIII. Mean[1] values of caffeine (%) extracted from whole guaraná seeds of Treatment 1[2].

Time (hours)	60% Ethanol -40% Water	40% Ethanol -60% Water	Methylene Chloride
4	$_{f}7.914^{d}$	$_{fg}5.840^{d}$	$_{g}4.275^{d}$
8	$_{f}29.700^{c}$	$_{g}9.576^{c}$	$_{g}9.302^{c}$
12	$_{f}33.103^{b}$	$_{g}15.037^{b}$	$_{h}13.609^{b}$
16	$_{f}34.013^{ab}$	$_{g}17.570^{a}$	$_{h}13.850^{b}$
20	$_{f}37.111^{a}$	$_{g}17.015^{a}$	$_{g}15.903^{a}$

[1] Means of 2 (60% ethanol-40% water) and 3 observations. Guaraná seeds contained a mean 4.07% caffeine (DWB).

[2] Chapter III, F.

[a-d] Means within a column followed by a different superscript letter are significantly different (0.05 level).

[f-h] Means within a row preceeded by a different subscript letter are significantly different (0.05 level).

hours. For the stated solvent, the difference between the amounts extracted at both times was not significant; however, there was a tendency for the amounts to increase after 16 hours.

The sum of squares from the analysis of variance of the values calculated from the regression curves is presented in Table XIV. The results indicate that at least one of the extraction curves of Figure 2 is significantly different (0.01 level) from the two other curves.

Figure 2 presents the curves for the amounts of caffeine (%) extracted by the three solvents according to the exponential models. The 60% ethanol-40% water solvent apparently extracted the greatest amount of caffeine. The amount of caffeine extracted with this solvent was approximately twice the amount extracted by either of the two other solvents after 20 hours.

Since solubility of caffeine in methylene chloride is approximately 5 times greater than that in ethanol and approximately 3.5 times greater than that in pure water (36), methylene chloride might be expected to be the most effective solvent. In this experiment methylene chloride extracted the least amount of caffeine from the seed. This phenomenon was discussed by Katz (29) who stated that the material that contains caffeine must pocess a minimum of 20% moisture for the extraction of caffeine; however, higher moisture levels

Table XIV. Sum of squares of the analysis of variance from values calculated from the regression of the curves of Treatment 1[1].

Source	df	Sum of Squares
Curves	4	2091.28**
Error (pooled)	38	63.59

[1] Chapter III, F.
** Significant at 0.01 level.

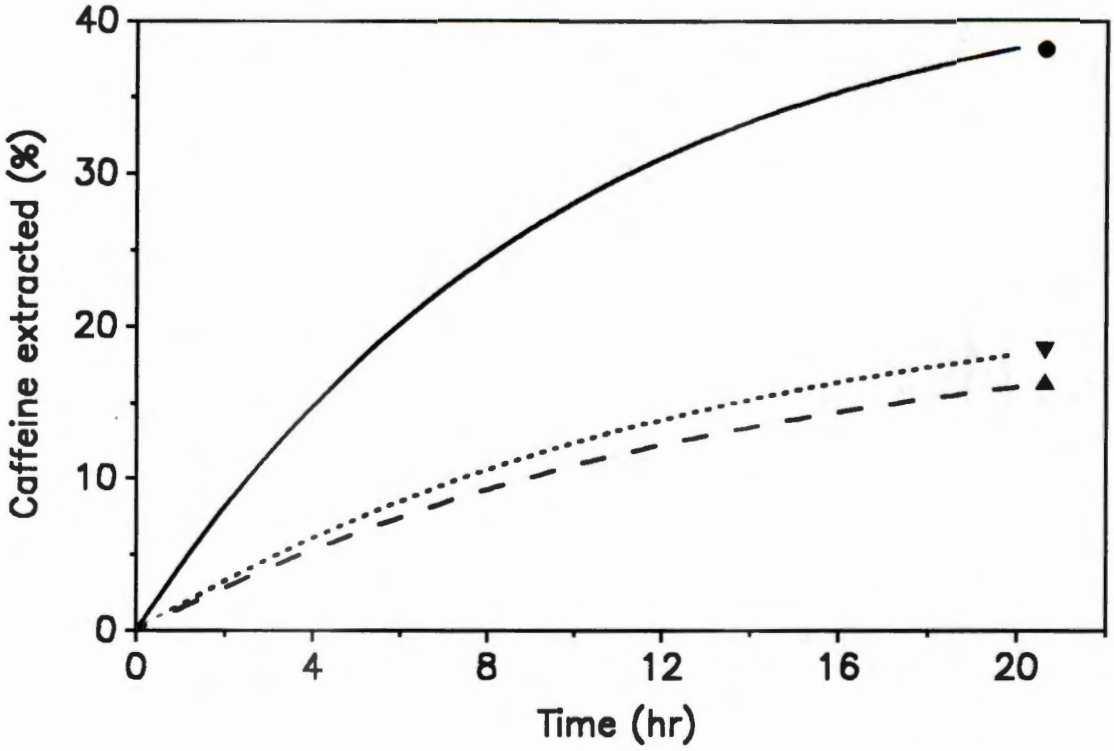

Figure 2. Curves for the amounts (%) of caffeine that should be extracted from whole guaraná seeds with the three solvents indicated, using the single-stage process with no agitation, Treatment 1 (Chapter III, F). Guaraná seeds contained a mean 4.07% caffeine (DWB).

●= 60% ethanol-40% water, ▼= 40% ethanol-60% water, and ▲= methylene chloride.

——● $\hat{y}$ = 43.672 (1-exp (-0.103 x)) (R^2 = 0.99)

·····▼ $\hat{y}$ = 23.281 (1-exp (-0.076 x)) (R^2 = 0.98)

- - -▲ $\hat{y}$ = 20.809 (1-exp (-0.074 x)) (R^2 = 0.99)

should be used to make the extraction economically feasible. Bichsel et al. (6) stated that the maximum water holding capacity of green coffee beans used in their experiments was 44-45%. Bichsel et al. (6) reported that only limited decaffeination can occur at a moisture content of below 20% regardless the amount of solvent used. Katz (29) explained that water softens the tissues, expands the cells, and allows caffeine to diffuse from the cells. Horman and Viani (27) suggested a more likely explanation that involved the ability of caffeine to bond with chlorogenic acid and its potassium chlorogenate salt to form a complex ion-salt-like material. The components formed are essentially insoluble in non-polar solvents.

The mean 17.02% (Table XIII) caffeine extracted by the 40% ethanol-60% water solvent after 20 hours provided an indication that a water content of 60% tended to decrease the capability of the solvent to extract caffeine in comparison to similar solvents of lower proportions of water. The amount of caffeine extracted under these conditions was approximately one-half that extracted by the 60% ethanol-40% water solvent. This finding indicates that relatively higher proportion of water in the solvent tends to reduce its capability to extract caffeine.

b. Treatment 2 - The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted from unprocessed seeds with the single-stage, constant agitation condition are presented in Table XV. The factors, time, solvent, and interaction had significant (0.01 level) effects on the percentage of caffeine extracted by the two solvents.

The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted by the two solvents within each time period and extracted at the six time periods within each solvent are presented in Tables XVI and XVII, respectively. Both factors, solvents within an individual time and times within an individual solvent, had significant (0.01 level) effect on the percentage of caffeine extracted with one exception; the factor, solvent within the eight hour extraction period, did not significantly (0.05 level) influenced extraction of caffeine.

The mean values of the amounts of caffeine (%) extracted from seeds are presented in Table XVIII. For each solvent the amount of caffeine extracted was increased with time. At the longest periods of time, 20 and 24 hours, the solvent, 60% ethanol-40% water, extracted the greater amount of caffeine. The amounts of caffeine extracted with this particular solvent did not significantly increase after 20 hours. Explanation for the difference in the amounts of caffeine extracted with the two solvents was presented in the discussion of Treatment 1.

Table XV. Sums of squares from the analysis of variance of caffeine (%) extracted from whole guaraná seeds with two solvents[1] over a 24-hour period[2] as described in Treatment 2[3].

Source	df	Sum of Squares
Time (T)	5	1978.04**
Solvent (S)	1	272.10**
T X S	5	309.31**
Error	25	17.90

[1] Solvents: 60% ethanol-40% water and methylene chloride.
[2] Time periods: 4, 8, 12, 16, 20, and 24 hours.
[3] Chapter III, F.
** Significant at 0.01 level.

Table XVI. Sums of squares from the analysis of variance of caffeine (%) extracted from whole guaraná seeds with the two solvents[1] within each time period of Treatment 2[2].

Source	df	Sum of Squares
Solvents within 4 hours	1	36.30**
Error	3	1.34
Solvents within 8 hours	1	3.23ns
Error	3	1.71
Solvents within 12 hours	1	456.03**
Error	3	0.57
Solvents within 16 hours	1	72.77**
Error	3	0.35
Solvents within 20 hours	1	207.34**
Error	3	0.93
Solvents within 24 hours	1	209.51**
Error	3	1.98

[1] Solvents: 60% ethanol-40% water and methylene chloride.
[2] Chapter III, F.
** Significant at 0.01 level.
ns Not significant at 0.05 level.

Table XVII. Sums of squares from the analysis of variance of caffeine (%) extracted from whole guaraná seeds at the six time periods[1] within each solvent of Treatment 2[2].

Source	df	Sum of Squares
Times within 60% Ethanol-40% Water	5	1601.81**
Error	6	4.02
Times within Methylene Chloride	5	456.46**
Error	12	2.86

[1] Time periods: 4, 8, 12, 16, 20, and 24 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XVIII. Mean[1] values of caffeine (%) extracted from guaraná seeds of Treatment 2^2.

Time (hours)	60% Ethanol -40% Water	Methylene Chloride
4	$_{h}7.982^{e}$	$_{g}13.482^{f}$
8	$_{g}22.515^{d}$	$_{g}20.875^{e}$
12	$_{g}33.103^{c}$	$_{h}23.609^{d}$
16	$_{g}33.015^{b}$	$_{h}25.229^{c}$
20	$_{g}40.530^{a}$	$_{h}27.385^{b}$
24	$_{g}41.935^{a}$	$_{h}28.722^{a}$

[1] Means of 2 (60% ethanol-40% water) and 3 observations. Guaraná seeds contained a mean 4.07% caffeine (DWB).
[2] Chapter III, F.
[a-f] Means within a column followed by a different superscript letter are significantly different (0.05 level).
[gh] Means within a row preceeded by a different subscript letter are significantly different (0.05 level).

A comparison of the data of Tables XIII and XVIII provided evidence that agitation was beneficial in the extraction of caffeine. For example, at the 20 hour period methylene chloride extracted approximately twice the amount of caffeine with agitation than was extracted with no agitation. However, the difference between the amounts of caffeine extracted with the 60% ethanol-40% water was less pronounced between conditions of agitation.

The sum of squares from the analysis of variance of the values calculated from the regression curves is presented in Table XIX. The results show that the curves in Figure 3 are different (0.01 level).

Figure 3 presents the curves for the amounts of caffeine (%) extracted by the two solvents according to the exponential models. The curves essentially overlaped between 0 and 8 hours. However, the curves began to separate after 8 hours and the difference increased with time.

Table XIX. Sum of squares of the analysis of variance from values calculated from the regression of the curves of Treatment 2[1].

Source	df	Sum of Squares
Curves	2	547.68**
Error (pooled)	28	21.76

[1] Chapter III, F.
** Significant at 0.01 level.

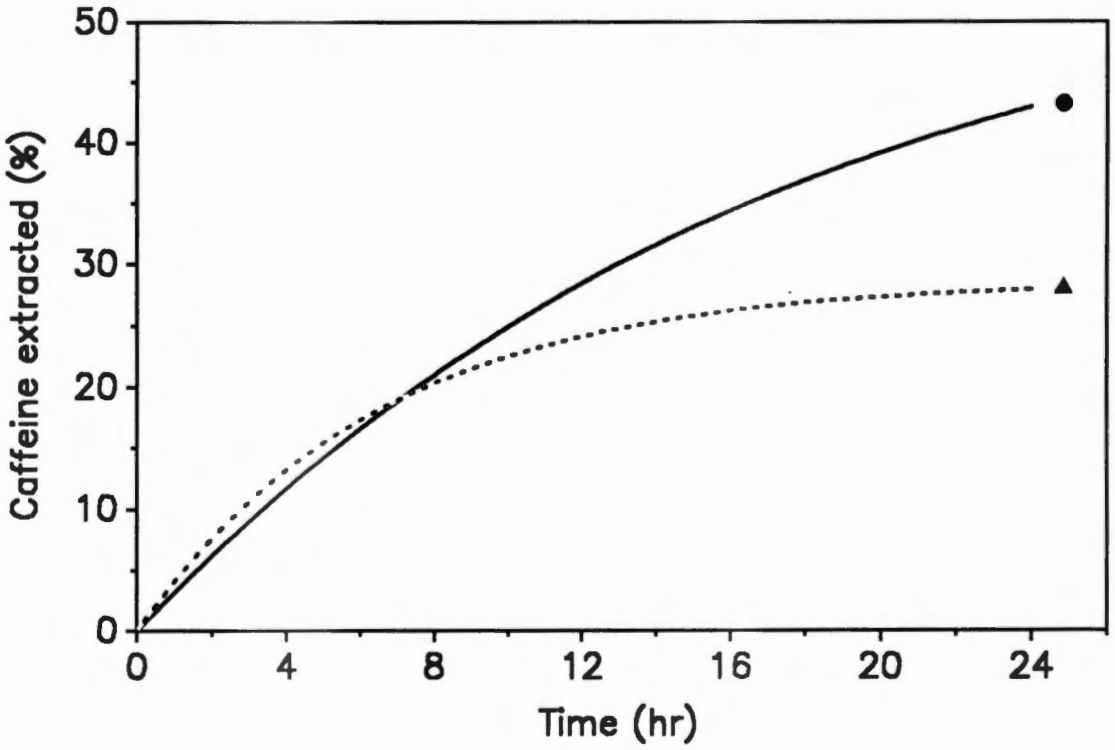

Figure 3. Curves for the amounts (%) of caffeine that should be extracted from whole guaraná seeds with the two solvents indicated, using the single-stage process with constant agitation, Treatment 2 (Chapter III, F). Guaraná seeds contained a mean 4.07% caffeine (DWB).

●= 60% ethanol-40% water, ▲= methylene chloride.

——● $\hat{y}$ = 57.945 (1-exp (-0.056 x)) (R^2 = 0.99)

·····▲ $\hat{y}$ = 28.600 (1-exp (-0.155 x)) (R^2 = 0.99)

c. Treatment 3 - The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted by methylene chloride from seeds that had been previously rehydrated in acid or alkaline solutions then held under conditions of single-stage, constant agitation are presented in Table XX. The factors, time, rehydration process, and interaction had significant (0.01 level) effect on the percentage of caffeine extracted.

The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted for the two rehydration processes within each time period and extracted at the five time periods within each rehydration process are presented in Tables XXI and XXII, respectively. Both factors, rehydration within an individual time and times within an individual rehydration, had significant (0.01 level) effect on the amounts of caffeine extracted with one exception; the factor, rehydration within the 4 hour period, was significant at the 0.05 level.

The mean values of the amounts of caffeine (%) extracted from the seeds are presented in Table XXIII. For the seeds rehydrated in alkaline solution the amounts of caffeine extracted increased with time. However, for the seeds rehydrated in acid solution the amounts of caffeine did not increase after the eight hour period.

Sivetz and Derosier (37) reported that extraction of

Table XX. Sums of squares from the analysis of variance of cafeine (%) extracted from whole guaraná seeds with methylene chloride over a 26-hour period[1]. The seeds were rehydrated in acid or alkaline solutions as described in Treatment 3[2].

Source	df	Sum of Squares
Time (T)	4	1005.99**
Rehydration (K)	1	1142.50**
T X K	4	636.31**
Error	20	5.08

[1] Time periods: 4, 8, 14 20,and 26 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XXI. Sums of squares from the analysis of variance of caffeine (%) extracted from rehydrated[1] seeds of guaraná with methylene chloride within each time period of Treatment 3[2].

Source	df	Sum of Squares
Rehydration within 4 hours	1	7.46*
Error	4	3.76
Rehydration within 8 hours	1	55.60**
Error	4	0.16
Rehydration within 14 hours	1	336.36**
Error	4	0.02
Rehydration within 20 hours	1	666.06**
Error	4	1.07
Rehydration within 26 hours	1	713.32**
Error	4	0.05

[1] Rehydration methods: acid and alkaline
[2] Chapter III, F.
* Significant at 0.05 level.
** Significant at 0.01 level

Table XXII. Sums of squares from the analysis of variance of caffeine (%) extracted from rehydrated seeds of guaraná with methylene chloride at the five time periods[1] within each rehydration process of Treatment 3[2].

Source	df	Sum of Squares
Times within rehydration with acid solution	4	22.09**
Error	10	3.92
Times within rehydration with alkaline soaking	4	1620.20**
Error	10	1.16

[1] Time periods: 4, 8, 14, 20, and 26 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XXIII. Mean[1] values of caffeine (%) extracted with methylene chloride from guaraná seeds of Treatment 3[2].

Time (hours)	Alkaline Rehydration	Acid Rehydration
4	$_{g}24.300^{e}$	$_{f}26.531^{b}$
8	$_{f}34.553^{d}$	$_{g}28.465^{a}$
14	$_{f}43.914^{c}$	$_{g}28.940^{a}$
20	$_{f}50.813^{b}$	$_{g}29.741^{a}$
26	$_{f}51.716^{a}$	$_{g}29.909^{a}$

[1] Means of 3 observations. Guaraná seeds contained a mean 4.07% caffeine (DWB).

[2] Chapter III, F.

[a-e] Means within a column followed by a different superscript letter are significantly different (0.05 level).

[fg] Means within a row preceeded by a different subscript letter are significantly different (0.05 level).

may involve preliminary preparation of the raw materials such as steaming, acid treatment, or alkaline treatment and that these preparations increase significantly (0.01 level) the yields of caffeine extracted. Indeed, alkaline rehydration resulted in a 2-fold increase in the amount of caffeine extracted at 20 hours as compared to the amount extracted at 4 hours. The reason for the greater extraction of caffeine from the seeds rehydrated in alkaline solution might be found in the research of Clifford (10). He found that caffeoylquinic acids (5-CQA) and probably many other chlorogenic acids (CGA) form complexes with caffeine and other alkaloids. Generally, the amount of CGA components in coffee beans is equivalent to the amount of caffeine, and such complexes that form between caffeine and CGA do occur naturally. Under temperatures above 80°C, the complex molecules are susceptible to attack by alkaline solutions and, thus, form soap-like-ion structures that are soluble in water. By this action, the caffeine molecule is released and can be extracted by the solvent. Conversely, acids in the rehydration process do not release caffeine, allowing it to remain insoluble and reducing the amounts of extractable caffeine.

The sum of squares from the analysis of variance of the values calculated from the regression curves is presented in Table XXIV. The results show that the curves in Figure 4 are different (0.01 level).

Table XXIV. Sum of squares of the analysis of variance from values calculated from the regression of the curves of Treatment 3[1].

Source	df	Sum of Squares
Curves	2	1819.88**
Error (pooled)	28	66.08

[1] Chapter III, F.
** Significant at 0.01 level.

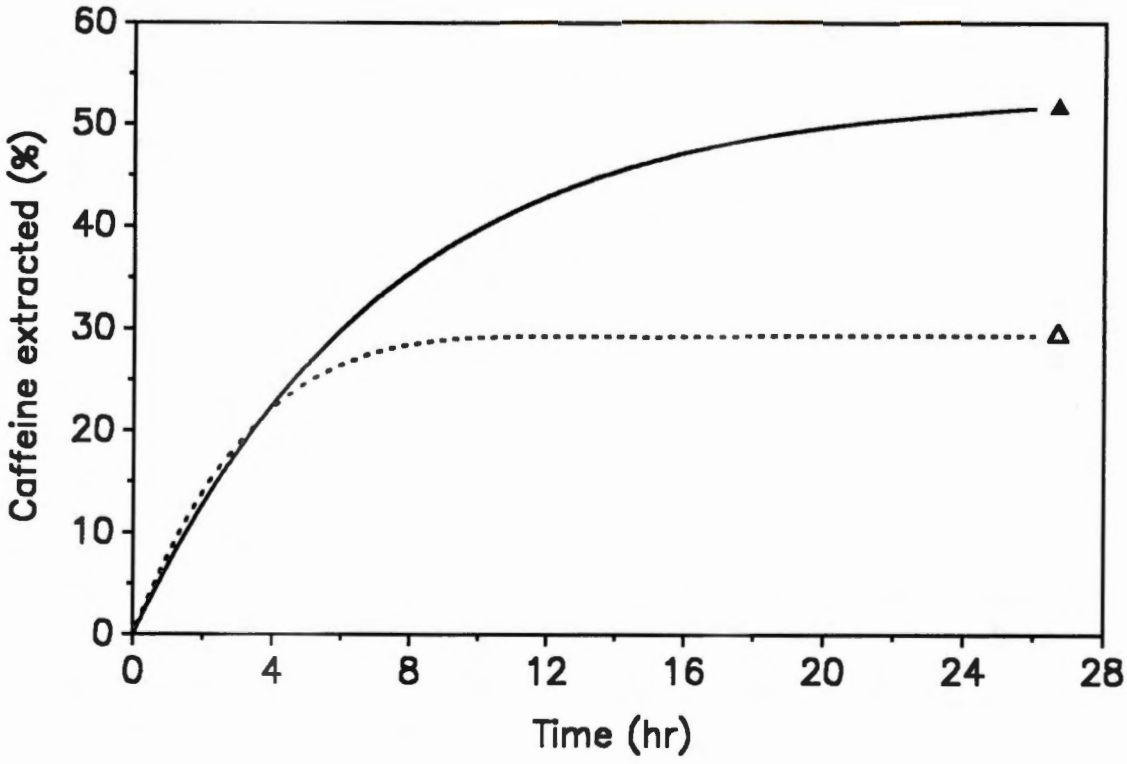

Figure 4. Curves for the amounts (%) of caffeine that should be extracted from whole guaraná seeds with methylene chloride, using the single-stage process with constant agitation. The seeds were rehydrated in acid or alkaline solutions, Treatment 3 (Chapter III, F). Guaraná seeds contained a mean 4.07% caffeine (DWB).

△= acid rehydration, ▲= alkaline rehydration.

·····△ $\hat{y}$ = 29.371 (1-exp (-0.573 x)) (R^2 = 0.99)

——▲ $\hat{y}$ = 53.039 (1-exp (-0.138 x)) (R^2 = 0.99)

Figure 4 presents the curves for the amounts of caffeine (%) extracted by the two solvents according to the exponential models. The curves overlaped between 0 and 4 hours. However, the curves began to separate after 4 hours and the difference between the curves increased with time. The curve representing the acid rehydrated process remained essentially unchanged after the 8 hour period.

2. Ground Seeds.

d. Treatment 4 - The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted with methylene chloride from ground guaraná seed material, employing the single-stage process with constant agitation or no agitation, are presented in Table XXV. The factors, time, agitation, and interaction had significant effect (0.01 level) on the percentage of caffeine extracted.

The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted with the two agitations within each time period and extracted at the seven time periods within each agitation are presented in Tables XXVI and XXVII, respectively. Both factors, agitations within individual times and times within individual agitations, had significant (0.01 level) effect on the percentage of caffeine extracted.

The mean values of the amounts of caffeine (%) extracted

Table XXV. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with methylene chloride, using constant agitation or no agitation over a 28-hour period[1] as described in Treatment 4[2].

Source	df	Sum of Squares
Time (T)	6	1324.12**
Agitation (A)	1	1419.06**
T X A	6	99.95**
Error	28	17.28

[1] Time periods: 4, 8, 12, 16, 20, 24, and 28 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XXVI. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with methylene chloride using agitation and no agitation within each time period of Treatment 4[1].

Source	df	Sum of Squares
Agitations within 4 hours	1	48.01*
Error	4	3.80
Agitations within 8 hours	1	105.79**
Error	4	2.82
Agitations within 12 hours	1	270.82**
Error	4	5.64
Agitations within 16 hours	1	333.07**
Error	4	1.08
Agitations within 20 hours	1	267.45**
Error	4	1.71
Agitations within 24 hours	1	270.83**
Error	4	1.11
Agitations within 28 hours	1	223.04**
Error	4	1.10

[1] Chapter III, F.
** Significant at 0.01 level.

Table XXVII. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with methylene chloride at the seven time periods[1] within each agitation method used with Treatment 4[2].

Source	df	Sum of Squares
Times within no agitation	6	391.96**
Error	14	5.11
Times within agitation	6	1032.12**
Error	14	12.16

[1] Time periods: 4, 8, 12, 16, 20, 24, and 28 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

from the seed material are presented in Table XXVIII. The amount of caffeine extracted with agitation was greater than the amount extracted with no agitation. In general, the amount of caffeine extracted without agitation increased with time. At 28 hours 24.5% caffeine had been extracted. With constant agitation the amount of caffeine extracted was increased up to 16 hours; thereafter, the amounts of caffeine extracted were not different.

The sum of squares from the analysis of variance of the values calculated from the regression curves is presented in Table XXIX. The results show that the curves in Figure 5 are different (0.01 level).

Figure 5 presents the curves for the amounts of caffeine (%) extracted with the two agitation processes according to the exponential models. The differences between the curves increased with time up to approximately 16 hours. After this period of time, the difference between the curves remained constant.

e. Treatment 5 - The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted from ground guaraná seed material with methylene chloride, using constant agitation, single-stage or simple multiple-stage processes are presented in Table XXX. The factors, time, stages, and interaction had significant (0.01 level) effect on the percentage of caffeine extracted. The sums of squares from the analysis

Table XXVIII. Mean[1] values of caffeine (%) extracted with methylene chloride from ground guaraná seed material of Treatment 4[2].

Time (hours)	Constant Agitation	No Agitation
4	$_{g}16.581^{d}$	$_{h}10.924^{f}$
8	$_{g}23.865^{c}$	$_{h}15.467^{e}$
12	$_{g}31.049^{b}$	$_{h}17.613^{d}$
16	$_{g}34.449^{a}$	$_{h}19.548^{c}$
20	$_{g}35.166^{a}$	$_{h}21.813^{b}$
24	$_{g}36.038^{a}$	$_{h}22.601^{b}$
28	$_{g}36.661^{a}$	$_{h}24.467^{a}$

[1] Means of 3 observations. Guaraná seeds contained a mean 4.07% caffeine (DWB).
[2] Chapter III, F.
[a-f] Means within a column followed by a different superscript letter are significantly different (0.05 level).
[gh] Means within a row preceeded by a different subscript letter are significantly different (0.05 level).

Table XXIX. Sum of squares of the analysis of variance from values calculated from the regression of the curves of Treatment 4[1].

Source	df	Sum of Squares
Curves	2	1500.42**
Error (pooled)	40	38.67

[1] Chapter III, F.
** Significant at 0.01 level.

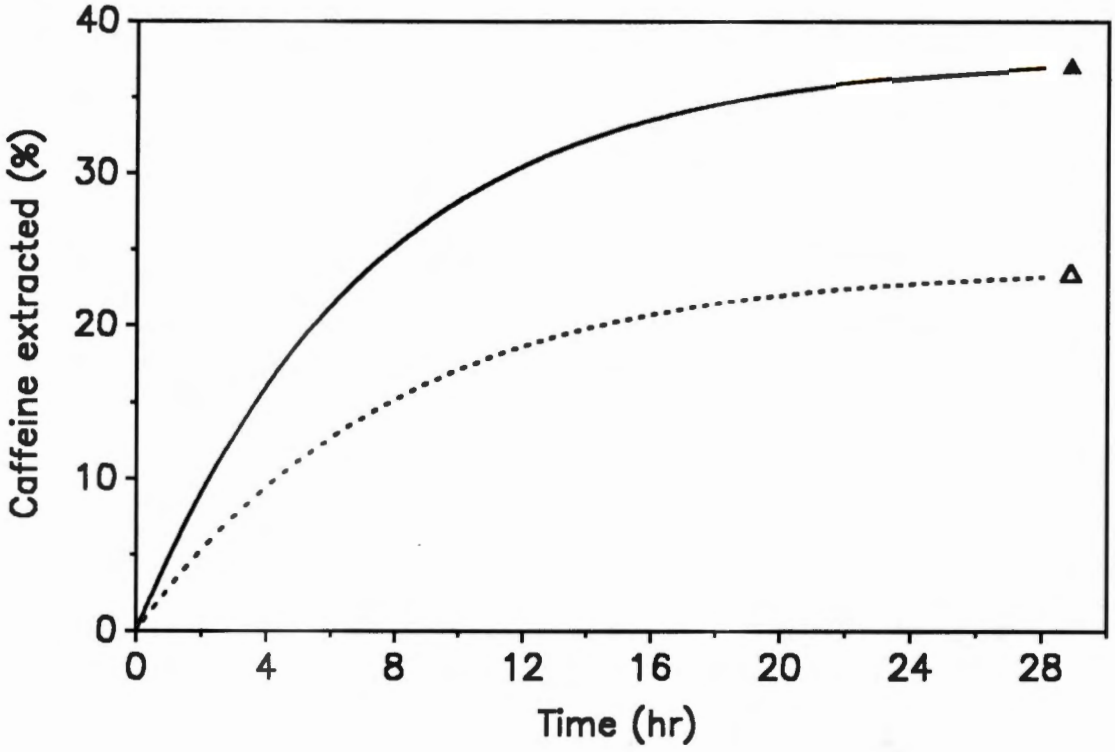

Figure 5. Curves for the amounts (%) of caffeine that should be extracted from ground guaraná seed material with methylene chloride, using the single-stage process with no agitation or constant agitation, Treatment 4 (Chapter III, F). Guaraná seed material contained a mean 4.07% caffeine (DWB).

Δ= no agitation, ▲= constant agitation.

·····Δ $\hat{y}$ = 23.874 (1-exp (-0.126 x)) (R^2 = 0.99)

——▲ $\hat{y}$ = 37.647 (1-exp (-0.138 x)) (R^2 = 0.99)

Table XXX. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with methylene chloride over a 30-hour period[1] using constant agitation, single-stage or multiple-stage processes of Treatment 5[2].

Source	df	Sum of Squares
Time (T)	4	2923.56**
Stage (G)	1	5725.08**
T X G	4	82.41**
Error	10	5.52

[1] Time periods: 6, 12, 18, 24, and 30 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

of variance of the amounts of caffeine (%) extracted with the two stages of extraction within each time period and extracted at the five time periods within each stage are presented in Tables XXXI and XXXII, respectively. Both factors, stages within an individual time and times within an individual stage, had significant (0.01 level) effect on the percentage of caffeine extracted.

The mean values of the amounts of caffeine (%) extracted from the seed material are presented in Table XXXIII. The amount of caffeine extracted with the simple multiple-stage was greater than the amount extracted with the single-stage process for which the amount of caffeine extracted was increased with time up to 30 hours. At this period approximately 68.5% of the caffeine had been extracted. With the multiple-stage process the amount of caffeine extracted was increased up to 24 hours with no difference found thereafter. After 24 hours 96.6% had been extracted . This value is essentially the same as that for commercially decaffeinated coffee (36).

The sum of squares from the analysis of variance of the values calculated from the regression curves is presented in Table XXXIV. The results show that the curves in Figure 6 are different (0.01 level).

Figure 6 presents the curves for the amounts of caffeine (%) extracted with the two stages, according to the exponential

Table XXXI. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with methylene chloride with single-stage and multiple-stage processes within each of the time periods of Treatment 5[1].

Source	df	Sum of Squares
Stages within 6 hours	1	867.89**
Error	2	3.89
Stages within 12 hours	1	1560.76**
Error	2	1.04
Stages within 18 hours	1	1394.05**
Error	2	0.07
Stages within 24 hours	1	1113.36**
Error	2	0.12
Stages within 30 hours	1	871.43**
Error	2	0.41

[1] Chapter III, F.
** Significant at 0.01 level.

Table XXXII. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with methylene chloride under constant agitation at the five time periods[1] within single-stage and within multiple-stage processes of Treatment 5[2].

Source	df	Sum of Squares
Times within single-stage	4	1446.33**
Error	5	3.88
Times within multiple-stage	4	1559.64**
Error	5	1.65

[1] Time periods: 6, 12, 18, 24, and 30 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XXXIII. Mean[1] values of caffeine (%) extracted with methylene chloride from ground guaraná seed material of Treatment 5[2].

Time (hours)	Single-stage Extraction	Multiple-stage Extraction
6	$_{f}34.633^{e}$	$_{g}64.093^{d}$
12	$_{f}47.223^{d}$	$_{g}86.729^{c}$
18	$_{f}57.250^{c}$	$_{g}93.587^{b}$
24	$_{f}63.265^{b}$	$_{g}96.632^{a}$
30	$_{f}68.534^{a}$	$_{g}98.054^{a}$

[1] Means of 2 observations. Guaraná seeds contained a mean 4.07% caffeine (DWB).
[2] Chapter III, F.
[a-e] Means within a column followed by a different superscript letter are significantly different (0.05 level).
[fg] Means within a row preceeded by a different subscript letter are significantly different (0.05 level).

Table XXXIV. Sum of squares of the analysis of variance from values calculated from the regression of the curves of Treatment 5[1].

Source	df	Sum of Squares
Curves	2	5799.32**
Error (pooled)	18	324.90

[1] Chapter III, F.
** Significant at 0.01 level.

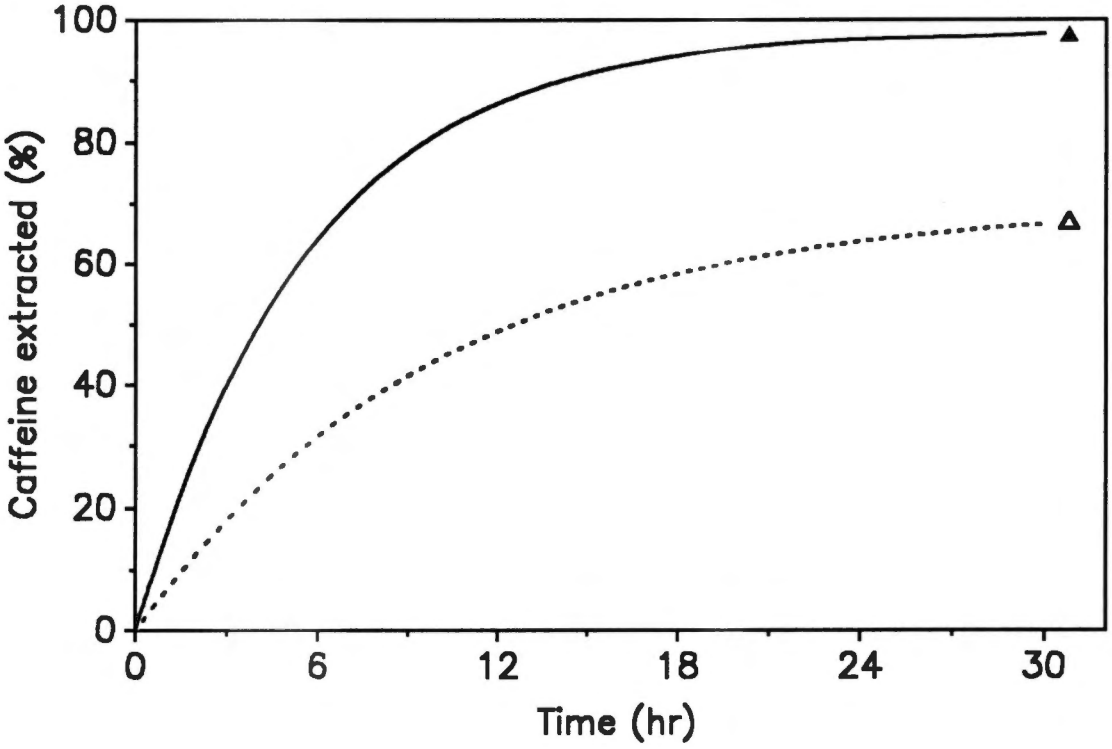

Figure 6. Curves for the amounts (%) of caffeine that should be extracted from ground guaraná seed material with methylene chloride, using the single-stage or the multiple-stage process with constant agitation, Treatment 5 (Chapter III, F). Guaraná seed material contained a mean 4.07% caffeine (DWB).

△= single-stage, ▲= multiple-stage

·····△ $\hat{y}$ = 70.076 (1-exp (-0.100 x)) (R^2 = 0.99)

——▲ $\hat{y}$ = 98.186 (1-exp (-0.176 x)) (R^2 = 0.99)

models. The differences between the curves increased with time up to approximately 8 hours. After this period of time, the difference between the curves remained constant.

f. Treatment 6 - The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted from ground seed material of guaraná with the simple multiple-stage, constant agitation condition are presented in Table XXXV. The factors, time, solvent, and interaction had significant (0.01 level) effect on the percentage of caffeine extracted.

The sums of squares from the analysis of variance of the amounts of caffeine (%) extracted with the three solvents within each time period and extracted at the five time periods within each solvent are presented in Tables XXXVI and XXXVII, respectively. Both factors, solvents within an individual time and times within an individual solvents, had significant (0.01 level) effect on the amount of caffeine extracted.

The mean values of the amounts of caffeine (%) extracted from the material are presented in Table XXXVIII. In general, the amount of caffeine extracted with the individual solvents increased with time up to 24 hours. The amounts of caffeine extracted at 24 hours with methylene chloride (96.6%) and ethyl acetate (93.8%) were not different (0.05 level), but these amounts were greater than the amount (77.2%) extracted

Table XXXV. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with three solvents[1] over a 30-hour period[2] using multiple-stage, constant agitation as described in Treatment 6[3].

Source	df	Sum of Squares
Time (T)	4	6347.62**
Solvent (S)	2	1822.41**
T X S	8	621.07**
Error	20	49.53

[1] Solvents: 95% ethanol-5% water, methylene chloride and ethyl acetate.
[2] Time periods: 6, 12, 18, 24, and 30 hours.
[3] Chapter III, F.
** Significant at 0.01 level.

Table XXXVI. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with the three solvents[1] within each of the time periods of Treatment 6[2].

Source	df	Sum of Squares
Solvents within 6 hours	2	374.56**
Error	4	5.10
Solvents within 12 hours	2	398.22**
Error	4	10.26
Solvents within 18 hours	2	485.01**
Error	4	10.71
Solvents within 24 hours	2	565.98**
Error	4	11.91
Solvents within 30 hours	2	619.70**
Error	4	11.55

[1] Solvents: 95% ethanol-5% water, methylene chloride and ethyl acetate.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XXXVII. Sums of squares from the analysis of variance of caffeine (%) extracted from ground guaraná seed material with the five time periods[1] within each of the three solvents of Treatment 6[2].

Source	df	Sum of Squares
Times within 95% Ethanol-5% Water	4	1338.14**
Error	10	34.72
Times within Methylene Chloride	4	1559.64**
Error	5	1.65
Times within Ethyl Acetate	4	3732.32**
Error	5	13.17

[1] Time periods: 6, 12, 18, 24, and 30 hours.
[2] Chapter III, F.
** Significant at 0.01 level.

Table XXXVIII. Mean[1] values of caffeine (%) extracted from guaraná seed material with the three solvents of Treatment 6[2].

Time (hours)	Methylene Chloride	Ethyl Acetate	95% Ethanol -5% Water
6	$_{f}64.093^{d}$	$_{h}44.861^{d}$	$_{g}52.819^{d}$
12	$_{f}86.729^{c}$	$_{g}71.846^{c}$	$_{g}69.135^{c}$
18	$_{f}93.587^{b}$	$_{g}87.447^{b}$	$_{h}74.364^{b}$
24	$_{f}96.632^{a}$	$_{f}93.846^{a}$	$_{g}77.194^{ab}$
30	$_{f}98.054^{a}$	$_{f}97.919^{a}$	$_{g}78.974^{a}$

[1] Means of 3 (95% ethanol-5% water) and 2 observations. Guaraná seeds contained a mean 4.07% caffeine (DWB).
[2] Chapter III, F.
[a-d] Means within a column followed by a different superscript letter are significantly different (0.05 level).
[f-h] Means within a row preceeded by a different subscript letter are significantly different (0.05 level).

by 95% ethanol-5%water. Presently, the Food and Drug Administration (FDA) is evaluating methylene chloride for its possible toxicity as a solvent for extracting caffeine (36). FDA permits decaffeinated coffee beans to contain 10 ppm methylene chloride residue instant coffee to contain 25 ppm of the residual solvent. With the use of other solvents such as super critical carbon dioxide and ethyl acetate, toxicity from the solvent can be eliminated (32, 38). Indeed, the present investigation showed that ethyl acetate is as effective for extracting caffeine of ground guaraná seed material as methylene chloride. In addition, the use of ethyl acetate is not a patented process (29). While the present cost of this solvent is somewhat greater than that of methylene chloride, there are indications that the cost of ethyl acetate will decrease in the future due to improvements in manufacturing.

The sum of squares from the analysis of variance of the values calculated from the regression curves is presented in Table XXXIX. The results indicate that at least one of the curves in Figure 7 is different (0.01 level) from the two other curves.

Figure 7 presents the curves for the amounts of caffeine (%) extracted by the three solvents, according to the exponential models. The 95% ethanol-5% water solvent apparently extracted the least amount of caffeine, based on the analyses

Table XXXIX. Sum of squares of the analysis of variance from values calculated from the regression of the curves of Treatment 6[1].

Source	df	Sum of Squares
Curves	4	2433.28**
Error (pooled)	33	608.32

** Significant at 0.01 level.
[1] Chapter III, F.

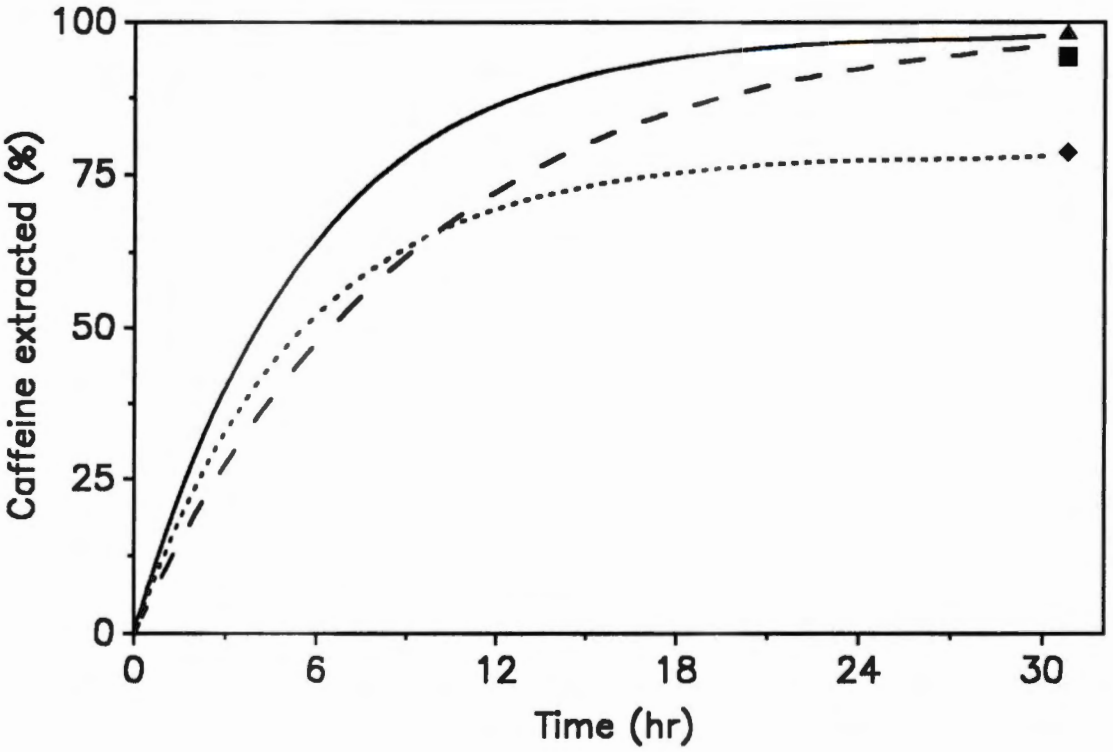

Figure 7. Curves for the amounts (%) of caffeine that should be extracted from ground guaraná seed material with the three solvents indicated, using the multiple-stage process with constant agitation, Treatment 6 (Chapter III, F). Guaraná seed material contained a mean 4.07% caffeine (DWB).

▲= methylene chloride, ■= ethyl acetate, ♦= 95% ethanol-5% water.

——▲ $\hat{y}$ = 98.186 (1-exp (-0.176 x)) (R^2 = 0.99)

- - -■ $\hat{y}$ = 100.000 (1-exp (-0.107 x)) (R^2 = 0.99)

·····♦ $\hat{y}$ = 78.228 (1-exp (-0.184 x)) (R^2 = 0.98)

presented in Table XXXIX. The curves that represent the two other solvents may be different; however, the caffeine amounts extracted with the two solvents at 24 hours are not.

E. Caffeine Diffusion from Guaraná Seeds and Ground Seed Material.

Extraction of caffeine from coffee beans and other similar materials using a solvent is a diffusion controlled rate process (41). According to Bichsel et al. (6) who reported on coffee, the calculation of the diffusion coefficient of caffeine is based on the following assumptions:

(1) the coffee beans are considered to be spheres;

(2) the diffusion is radial, isotropic and independent of the concentration of the caffeine in the beans;

(3) at the beginning of the experiment (t=0) the caffeine is evenly distributed in the beans; and

(4) during the whole experiment the caffeine content of the extract phase equals zero.

In this investigation, the same assumptions were considered for guaraná.

The longitudinal and latitudinal diameters of guaraná seed ranged from 0.82 to 1.39 cm and from 0.83 cm to 1.25 cm, respectively. The mean values found for the latitudinal and longitudinal diameters were 1.11 cm ± 0.42 and 1.04 cm ± 0.35, respectively. The mean radius calculated from equation 1 (Chapter III, C) was 0.54 cm.

Figures 8, 9, and 10 represent the rates of diffusion of caffeine from whole guaraná seeds. Figure 8 refers to the

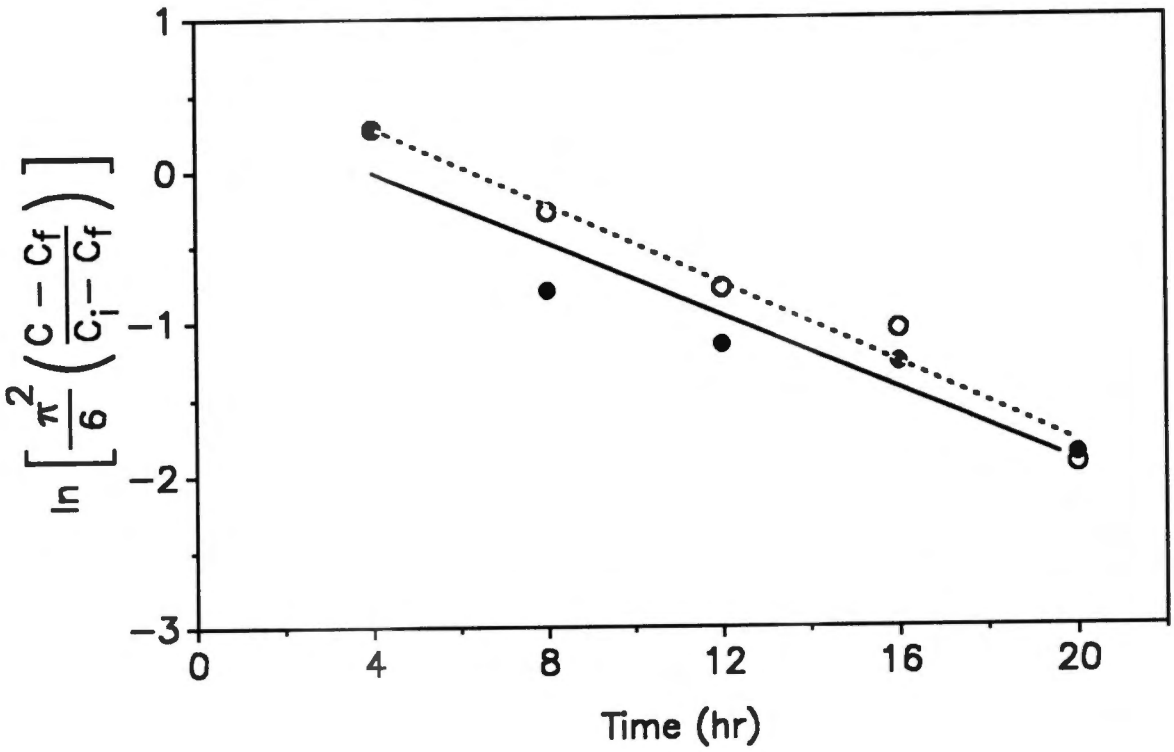

Figure 8. The curves represent the rate of diffusion of caffeine from whole guaraná seed extracted with 60% ethanol-40% water, using the single-stage process with no agitation or constant agitation (Chapter III, G).
o= no agitation, •=constant agitation.

..o.. $\hat{y} = -0.121\ x$ ($r = -0.98$)

__•__ $\hat{y} = -0.108\ x$ ($r = -0.98$)

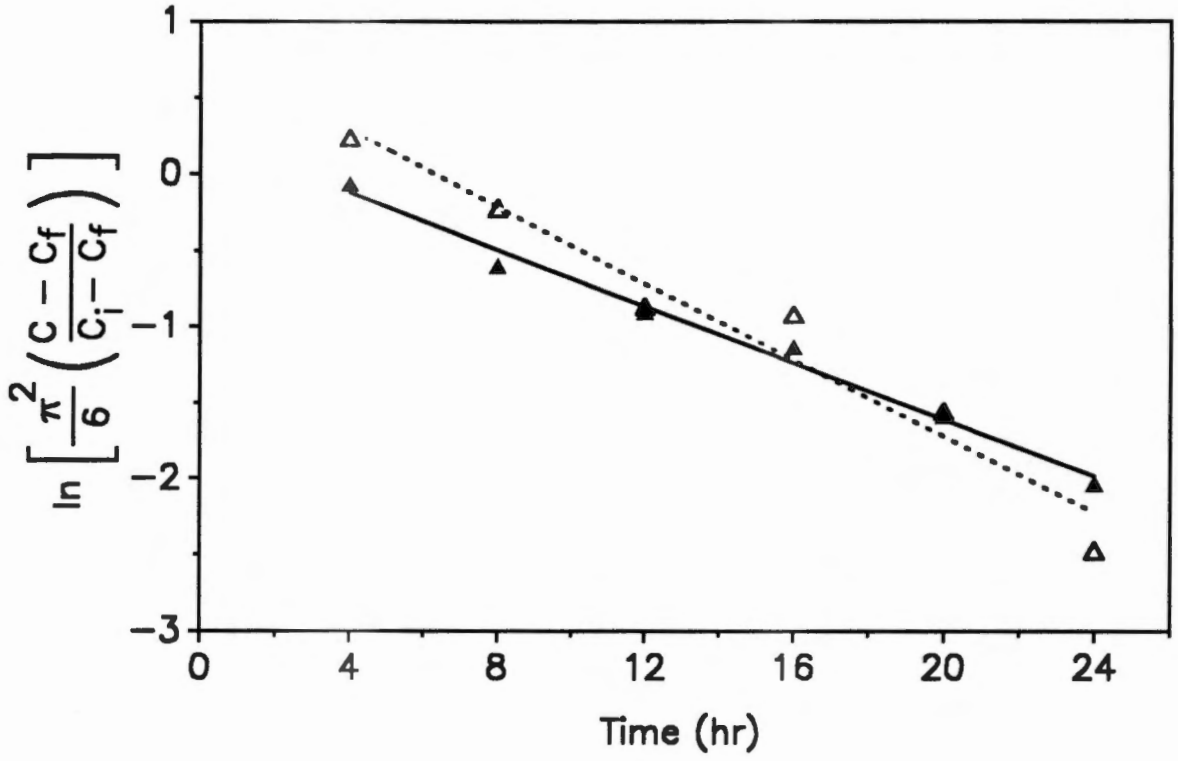

Figure 9. The curves represent the rate of diffusion of caffeine from whole guaraná seed extracted with methylene chloride, using the single-stage process with no agitation or constant agitation (Chapter III, G).

△= no agitation, ▲= constant agitation.

..△.. $\hat{y} = -0.109\ x$ ($r = -0.98$)

__▲__ $\hat{y} = -0.107\ x$ ($r = -0.99$)

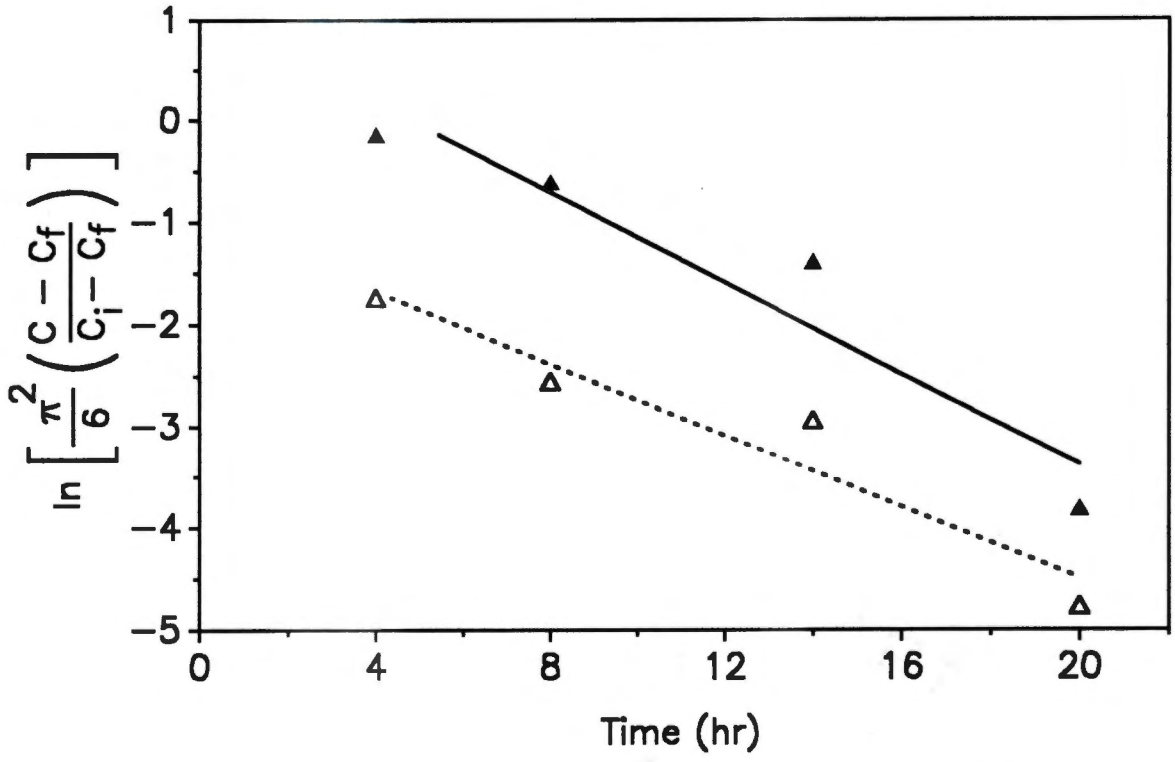

Figure 10. The curves represent the rate of diffusion of caffeine from whole guaraná seed extracted with methylene chloride, using the single-stage process with acid or alkaline rehydration and with constant agitation (Chapter III, G).

△= acid rehydration, ▲= alkaline rehydration.

..△.. $\hat{y} = -0.276\ x$ ($r = -0.95$)

___▲___ $\hat{y} = -0.183\ x$ ($r = -0.95$)

extraction with 60% ethanol-40% water, using the single-stage process with constant agitation or no agitation. Based on these conditions of extraction the diffusion coefficient (D) for the process with no agitation was found to be 0.66×10^{-6} cm^2/sec. For the process under agitation condition, $D = 0.61 \times 10^{-6}$ cm^2/sec. Thus, the diffusion of caffeine was greater when no agitation was used. No explanation for this finding is available.

Figure 9 refers to the extraction with methylene chloride, using the single-stage process with constant agitation or no agitation. For the process with no agitation, $D = 0.73 \times 10^{-6}$ $cm^2/sec.$; for the process with agitation, $D = 0.74 \times 10^{-6}$ cm^2/sec.

Figure 10 presents the rates of diffusion of caffeine in seeds that had been rehydrated in acid or alkaline solutions. The single-stage process with constant agitation was used. For seeds treated with acid solution, $D = 0.29 \times 10^{-6}$ $cm^2/sec.$, and for seeds treated with alkaline solution, $D = 0.43 \times 10^{-6}$ cm^2/sec. Thus, the rate of diffusion was greater for the alkaline-treated seeds.

Bichsel et al. (6) reported the apparent coefficients of diffusion for green coffee beans as affected by temperature. The coefficients ranged from $0.24 - 0.89 \times 10^{-6}$ cm^2/sec.

Figures 11, 12, and 13 represent the rates of diffusion of caffeine from guaraná seed material of particle sizes

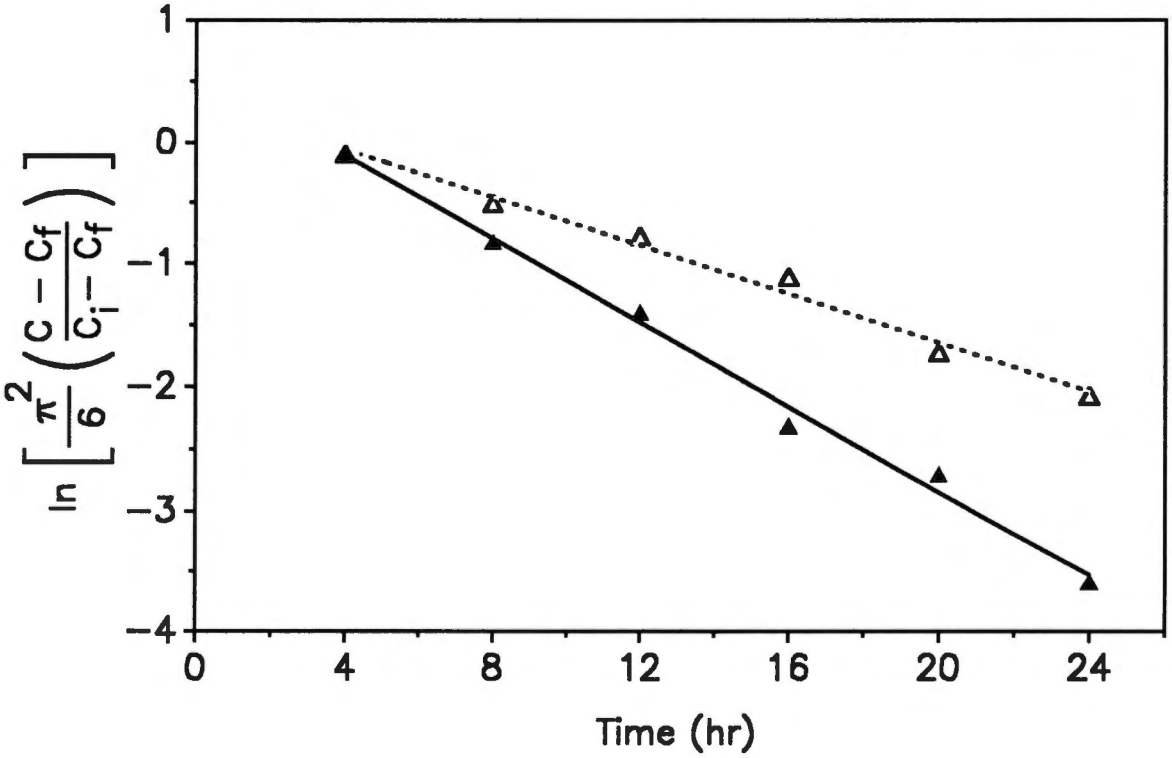

Figure 11. The curves represent the rate of diffusion of caffeine from ground guaraná seed material extracted with methylene chloride, using the single-stage process with no agitation or constant agitation (Chapter III, G). △= no agitation, ▲= constant agitation.

..△.. $\hat{y} = -0.108\ x$ ($r = -0.99$)

___▲___ $\hat{y} = -0.167\ x$ ($r = -0.99$)

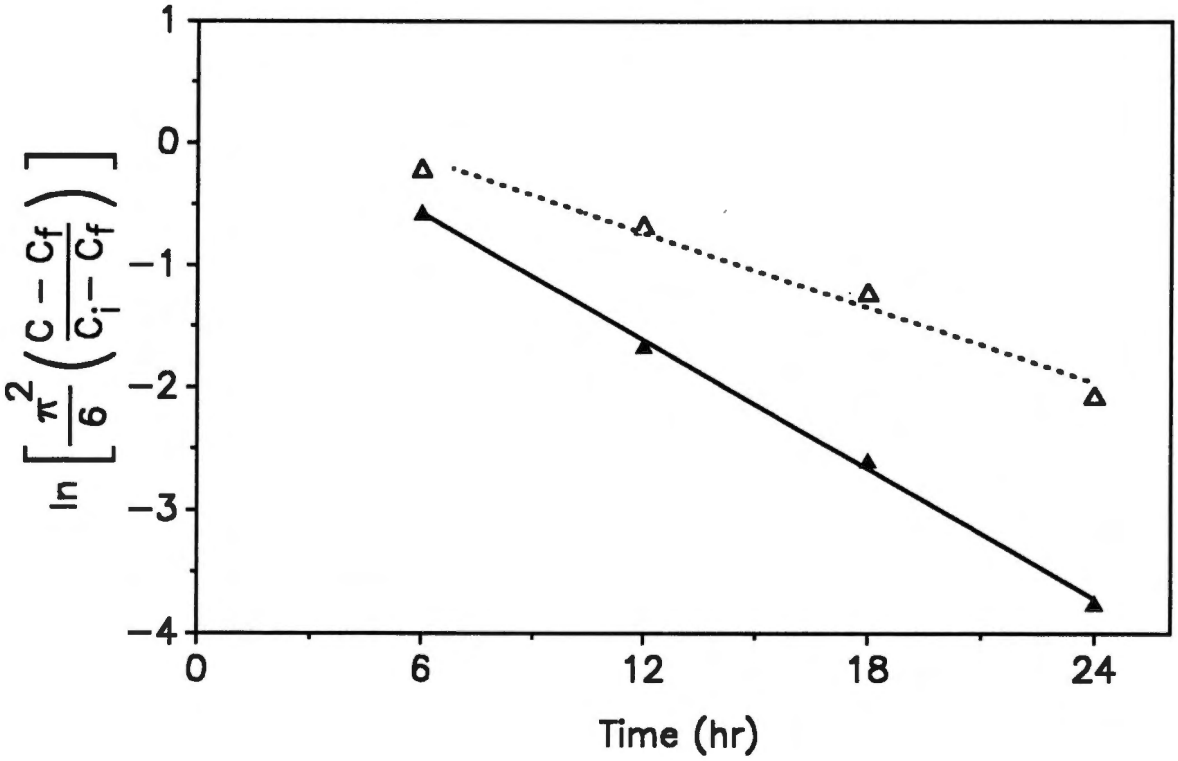

Figure 12. The curves represent the rate of diffusion of caffeine from ground guaraná seed material extracted with methylene chloride, using the single-stage or the multiple-stage process with constant agitation (Chapter III, G).

△= single-stage, ▲= multiple-stage.

..△.. $\hat{y} = -0.102\ x$ ($r = -0.99$)

__▲__ $\hat{y} = -0.176\ x$ ($r = -0.99$)

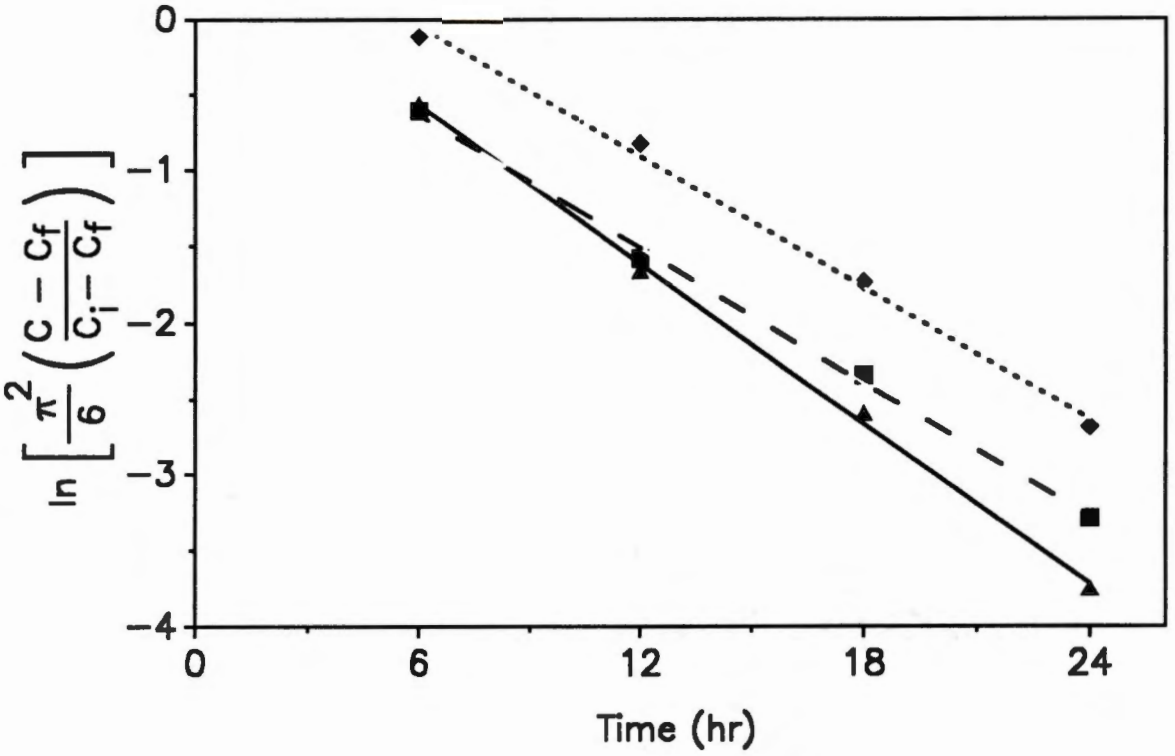

Figure 13. The curves represent the rate of diffusion of caffeine from ground guaraná seed material extracted with methylene chloride, ethyl acetate, or 95% ethanol-5%water, using the multiple-stage process with constant agitation (Chapter III, G).

▲= methylene chloride, ■= ethyl acetate, ♦= 95% ethanol-5% water.

▲ $\hat{y} = -0.176\ x$ (r = -0.99)

■ $\hat{y} = -0.126\ x$ (r = -0.99)

♦ $\hat{y} = -0.160\ x$ (r = -0.99)

presented in Chapter III, D. 2. Figure 11 refers to the extraction with methylene chloride, using the single-stage process with constant agitation or with no agitation. For the agitation method, $D' = 0.46 \times 10^{-4}$ sec.$^{-1}$, and for the method with no agitation, $D' = 0.30 \times 10^{-4}$ sec.$^{-1}$. Agitation increased the diffusion of caffeine from the material.

Figure 12 refers to the extraction with methylene chloride from seed material that had been rehydrated in alkaline solution, using the single-stage or multiple-stage process, both with constant agitation. $D' = 0.28 \times 10^{-4}$ sec.$^{-1}$ for the single-stage process, and $D' = 0.49 \times 10^{-4}$ sec.$^{-1}$ for the multiple-stage process. The latter process improved the diffusion of caffeine under the stated conditions.

Figure 13 refers to the extraction with methylene chloride, ethyl acetate, or 95% ethanol-5% water from seed material that had been rehydrated in alkaline solution, using the multiple-stage process with constant agitation. For methylene chloride $D' = 0.49 \times 10^{-4}$ sec.$^{-1}$, for ethyl acetate $D' = 0.45 \times 10^{-4}$ sec.$^{-1}$, and for 95% ethanol-5% water $D' = 0.35 \times 10^{-4}$ sec.$^{-1}$. Thus, the effect of methylene chloride and ethyl acetate were similar and for both solvents the diffusion was greater than that for the 95% ethanol-5% water solvent.

F. Alkaloids Distribution on Guaraná Leaves.

Only one clone (# 217) of the 17 clones of guaraná tested, contained caffeine in leaves (0.88%). However, leaves for all clones contained theobromine at a range of 0.07 to 0.58% and a mean 0.18%. No theophylline was found. These results are in agreement with those reported by BRAHMA (7). The mean values of the amounts of theobromine present in the leaves of guaraná were different (0.01 level). The amounts of theobromine detected were considered small and no further efforts were made to evaluate the potentiality of this material to be used as a natural source of alkaloids.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objectives of this research were to determine the most appropriate treatments and conditions for extracting caffeine from guaraná seed and to determine the most effective solvents to accomplish the extraction.

Seeds of guaraná, a Brazilian fruit (*Paulinia cupana* var. *sorbilis* (Mart.) Ducke), were used as the source of caffeine. The seeds were prepared under various conditions to increase the yield of extraction of caffeine. Preparations included rehydrating the seed or ground seed material in acid or alkaline solutions to 40% moisture and steaming for 30 minutes. Single-stage (solvent was not replaced with fresh solvent during the extraction period) or multiple-stage (solvent was replaced with fresh solvent after specified periods of time) processes were used with and without agitation to extract caffeine. The solvents were ethanol-water mixtures of different proportions, methylene chloride, and ethyl acetate.

Analyses of the seeds were conducted to measure the composition of proximate components, minerals,and caffeine and to identify volatiles extracted with methylene chloride. The treated seeds and seed material were extracted with the

solvents, and the percentage of caffeine extracted for up to 30 hours was measured. The data were used to calculate the coefficients of diffusion of caffeine.

From this study, the following conclusions may be made:

1. Seeds dried on iron plates held over clay ovens (traditional drying method) contained higher amounts of all proximate components than seeds dried by solar radiation, except for carbohydrate. Calcium, potassium, copper, and iron contents were higher in the traditionally dried seeds.

2. The traditionally dried seeds contained a mean 4.07% caffeine (DWB), and the seeds that were dried by solar radiation contained 4.40% (DWB). Because of the much greater availability of traditionally dried seeds, all the experiments on extraction of caffeine were conducted on this type of seed.

3. A diverse group of 15 volatile components was identified tentatively.

4. Rehydration of the seeds and reduction of seeds to ground material increased the extraction of caffeine. As an example, seeds rehydrated with alkaline solution yielded 1.9 times more caffeine than dry seeds after methylene chloride extraction for 20 hours.

5. Agitation increased the extracted yield of caffeine from both, seeds and ground material. For example, the amount of caffeine extracted with methylene chloride from ground seed material under agitation was 1.6 times greater than that

extracted without agitation.

6. A greater amount of caffeine was extracted with methylene chloride from seeds rehydrated with alkaline solution than from seeds rehydrated with acid solution. After 26 hours of extraction, alkaline rehydrated seeds yielded 1.7 times more caffeine than acid rehydrated seeds.

7. Greater amounts of caffeine were extracted with methylene chloride, using the multiple-stage process.

8. Alkaline rehydrated, ground seed material yielded greater amounts of caffeine than alkaline rehydrated, whole seeds.

9. It is concluded from this experiment that the following conditions should be applied for extracting the maximum amounts of caffeine from guaraná seeds or seed material: rehydrate the materials with alkaline solution, use methylene chloride as extraction agent, apply constant agitation, and replace solvent after specified periods of time. Ethyl acetate may be used as an alternative solvent when its cost becomes competitive with that of methylene chloride.

10. The coefficients of diffusion reflected the effect of the test conditions on the rate of caffeine extraction from guaraná seeds and ground seed material. These coefficients were within the range of those reported for coffee.

11. With one exception (clone # 217), leaves of the guaraná plant contained, at most, only traces of caffeine.

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APPENDIX

Equation (4) in Udaya Sankar et al. (41) is

$$\ln\left[\frac{\pi^2}{6}\left(\frac{C-C_f}{C_i-C_f}\right)\right] = \left(-\frac{1}{\tau}\right)t .$$

Substituting $\tau = \frac{r_o^2}{\pi^2 D}$ in the previous equation one gets

$$\ln\left[\frac{\pi^2}{6}\left(\frac{C-C_f}{C_i-C_f}\right)\right] = -\left(\frac{D\pi^2}{r_o^2}\right)t ,$$

or,

$$\ln\left[\frac{\pi^2}{6}\left(\frac{C-C_f}{C_i-C_f}\right)\right] = -D' t ,$$

where $D' = \frac{\pi^2 D}{r_o^2}$.

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