Analysis of Total Caffeine and Other Xanthines in Specialty Coffees Using Mixed Mode Solid-Phase Extraction and Liquid Chromatography–Diode-Array Detection After Microwave Digestion

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Abstract

In this study, solid-phase extraction (SPE) in mixed mode operation was employed to isolate xanthines including caffeine and theobromine from milled caffeinated and decaffeinated coffee samples after microwave digestion. 8-Chlorotheophylline was used as an internal standard. SPE was performed in hydrophobic mode using ethyl acetate/methanol (90:10, 2 mL) as the first elution solvent and in ionic exchange mode using ethyl acetate/acetonitrile/ammonium hydroxide (78:20:2, 3 mL) as the second elution solvent. The eluates were combined, evaporated to dryness and dissolved in aqueous formic acid for analysis. Liquid chromatography with photodiode-array detection was used in isocratic mode employing a C_{18} column and a mobile phase consisting of acetonitrile/formic acid (0.1% aqueous). The limits of quantitation and detection for this method were 1 and 0.1 mg/L, respectively. The method was linear from 1 to 200 mg/L ($r^2 > 0.999$) with recoveries of the individual xanthines greater than 95%. The decaffeinated coffees contained caffeine at levels less than 0.5 mg/g (range 0.23 to 0.49 mg/g) and caffeinated samples had wide range of levels of caffeine (5.18 to 12.21 mg/g).

Introduction

Ever since the days of the famous explorer Sir Walter Raleigh, when coffee was brought to Europe, caffeine has enjoyed the position of being one of the world's most popular drugs. Caffeine, theobromine, and theophylline (Figure 1) belong to a group of compounds known as the xanthines. Caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) (1) are in such beverages as coffee, teas and colas and are common to the urine of these drinkers (2). Theobromine is the major xanthine in chocolate (3). Theophylline (1,3-dimethylxanthine) should be absent from legitimate coffee beverages, although it does exist in the leaves of teas (1). The metabolism of caffeine in the human system involves the formation of both theobromine and theophylline (1). A method for differentiating unauthorized employment of theophylline and normal caffeinated beverages has been reported in the literature (4).

Caffeine, theobromine, and theophylline possess the properties of mild central nervous system stimulants. Caffeine also

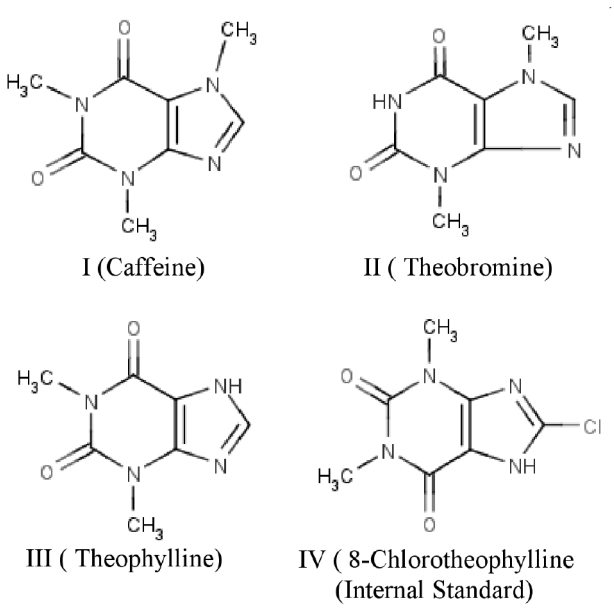

Figure 1. Structures of caffeine (I), theobromine (II), theophylline (III), and the internal standard 8-chlorotheophylline (IV).

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acts as a diuretic, and it may produce myocardial and respiratory stimulation. The effects of theobromine and theophylline are less pronounced than those of caffeine (1). It has been reported that therapeutic blood levels of caffeine and theophylline are 2–10 mg/L and 10–20 mg/L, respectively (5). Fatal blood levels of caffeine range from 79 to 344 mg/L (1), and for theophylline, the blood levels in fatal cases have ranged from 63 to 250 mg/L (6) There are, at present, no published data regarding fatalities directly attributable to overdosing on theobromine (as a singular drug) in databases such as Pubmed or Science Direct.

Studies have been reported regarding the concentrations of available caffeine in samples of specialty beverages obtained from retail outlets (7,8). These reports concerning both caffeinated and decaffeinated coffee samples have employed liquid–liquid extraction followed by gas chromatography and nitrogen-phosphorus detection (GC–NPD) in the analysis. The determination of xanthines in both beverages and biological samples has also been previously reported using thin-layer chromatography (9), liquid chromatography (LC) (10), and mass spectrometry (MS) (11).

With all this information available to the analyst in a forensic toxicological laboratory, the question still remains how much caffeine is present in the original specialty coffee beans? This study was initiated with the idea of developing a method to answer this question and determine how much (if any) of the other xanthines are present.

Liberation of xanthines from aqueous extracts of powdered coffee bean samples was undertaken with the aid of a domestic microwave unit. The analytical procedure was performed using mixed mode solid-phase extraction (SPE) and LC–diode-array detection rather than the previously noted GC–NPD. The data gained from this study in terms of methodology and data add to the body of knowledge regarding concentrations of xanthines in coffee (especially caffeine) available for consumption by humans.

Experimental

Reagents and materials

Caffeine, theobromine, and theophylline were obtained from Sigma-Aldrich (St. Louis, MO), and 8-chlorotheophylline was obtained from Acros Chemicals (via Fisher Scientific, Pittsburgh, PA). All materials were supplied as solid powders. Methanolic solutions of caffeine and theophylline (1 g/L) and theobromine (0.1 g/L) were also obtained from Cerilliant (Round Rock, TX). Acetonitrile, ammonium hydroxide, ethyl acetate, formic acid, and methanol were obtained from Fisher Scientific. Glacial acetic acid was obtained from Mallinckrodt Chemicals (Phillipsburg, NJ). All chemicals were of ACS grade unless otherwise stated. SPE columns (CSDAU, 6 mL, 200 mg) were obtained from United Chemical Technologies (UCT, Bristol, PA).

A 0.1 M acetic acid solution was made by diluting 2.8 mL of glacial acetic acid into 500 mL with distilled water.

Caffeinated and decaffeinated coffee beans were obtained in Philadelphia, PA (Phil), and in Brisbane, Australia (Bris).

Selection of internal standard

In the papers noted earlier by McCusker et al. (7,8), mepivicaine was employed as an internal standard. This is a compound that is often found in forensic laboratories undertaking toxicological analysis of basic drugs in biological samples. Mepivicaine is a suitable internal standard when used with GC–NPD systems and with MS. Selection of a suitable alternative to mepivicaine (which is not a xanthine) for LC was based on finding a xanthine type compound that more closely resembled caffeine, theobromine, and theophylline. It needed to have the ability to be introduced into samples and be extracted quantitatively as well as be resolved from the xanthines (caffeine, theobromine, and theophylline) chromatographically. The suitable candidate had to be available commercially in a pure state and could not be a compound observed in coffees. The compound that fulfilled all of those requirements was 8-chlorotheophylline.

Instrumentation

SPE was carried out using a 24-position vacuum manifold supplied by UCT. High-performance liquid chromatography (HPLC) was performed on an Agilent Technologies (Wilmington, DE) series 1100 system comprising of G1322A degasser, G1311A quaternary pump, G1313A ALS autosampler, and G1315A DAD unit. Separation was carried out on a Gold C_{18} column (C_{18}, 4.6 × 150 mm, 5 µm) supplied by ThermoFisher (Bellefonte, PA); this was attached to a guard column cartridge unit (Allure Basix, 10 × 2.1 mm) supplied by Restek (Bellefonte, PA). The mobile phase was operated isocratically using acetonitrile/0.1% formic acid in distilled water (10:90 v/v) at 0.1 mL/min throughout the analysis,

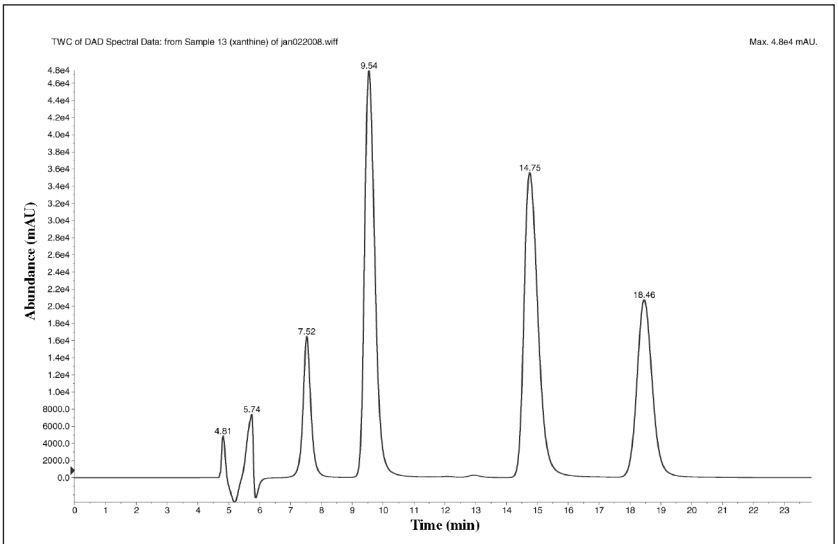

Figure 2. Chromatogram of xanthines (200 mg/L). Elution order: theobromine (Rt = 7.5 min), theophylline (Rt = 9.5 min), caffeine (Rt = 14.7 min), and 8-chlorotheophylline (IS) (Rt = 18.4 min).

and the injection volume was 10 µL. Chromatographic analysis was performed at room temperature. The photodiode-array detector was set to record over the range 200 to 350 nm. A lambda maxima (λ_{max}) value of 272 nm was used for all analytes. Data were collected using Analyst software version 1.4.2 obtained from Applied Biosystems (Foster City CA).

Sample pretreatment: calibrators and controls

Solutions of caffeine, theobromine, theophylline, and 8-chlorotheophylline (1 g/L) were each prepared by dissolving 10 mg of the powder with 10 mL of methanol in individual volumetric flasks and these were used as calibrators.

Control samples were prepared from solutions obtained from a separate source. These controls were prepared at 7, 75, and 150 µg with respect to caffeine. The theobromine control was prepared at a level of 7 µg.

Chromatographic analysis

Chromatographic separation conditions were developed by injection of solutions of each of the xanthines separately into the LC. The proportion of each component (acetonitrile/0.1% aqueous formic acid) in the mobile phase was adjusted until baseline resolution of each of the xanthines was achieved. The retention times for each analyte were also noted. The chromatography of the xanthines was adjusted so that the first component (theobromine) was eluted after 5 min, and the subsequent components (theophylline, caffeine, and 8-chlorotheophylline) were baseline separated. The chromatographic run time for each analysis was set at 25 min. The UV spectrum for each component was also recorded. A chromatogram of the resolved xanthines is shown in Figure 2.

Sample pretreatment: coffee grinding

Powdered coffee was produced by grinding approximately 30 g of each of the specialty coffees listed in Table I. The coffee beans were ground to a fine powder with a Cuisinart model DCG-123C coffee grinder. The coffee beans were placed into the instrument and ground for approximately 5 min. After this time, the contents of the grinder were emptied into a plastic sealable bag and labeled with an identifier. To ensure that the grinder was free from contamination, it was then cleaned of any residual coffee powder with a brush and visually inspected to ensure it was free of coffee before the next sample was powdered

Microwave digestion

Into a clean, dry 400-mL beaker was added approximately 1 g of powdered coffee in duplicate. The average mass of sample taken for caffeinated coffees was 1.0420 ± 0.02 g (n = 64), and

Table I. Xanthine Levels for Analyzed Coffee Samples

Coffee	Country of Origin	City	Caffeine (mg/g)	Theobromine (mg/g)	Theophylline (mg/g)
Caffeinated Coffees					
French Coffee	USA*	Phil	7.84	0.00	0
Italian Caffeine	USA	Phil	9.76	0.00	0
Guatamala Antigua	USA	Phil	11.27	0.00	0
Sulawesi Caffeine	USA	Phil	10.54	0.00	0
Sumatra Caffeine	USA	Phil	7.63	0.00	0
Organic Shade Mexican Caffeine	USA	Phil	8.15	0.00	0
House Caffeine	USA	Phil	10.45	0.00	0
Serena Caffeine	USA	Phil	9.00	0.00	0
Café Estima	USA	Phil	8.92	0.00	0
Gold Coast Caffeine	USA	Phil	10.72	0.00	0
Verona Caffeine	USA	Phil	11.11	0.00	0
Expresso Caffeine	USA	Phil	10.19	0.00	0
Italian Roast Caffeine	Aus	Bris	7.74	0.00	0
Kenya Caffeine	Aus	Bris	7.05	0.00	0
Xmas Blend Caffeine	USA	Phil	7.29	0.00	0
Xmas Blend Caffeine	USA	Phil	12.21	0.00	0
Kona Caffeine	USA	Phil	5.18	0.00	0
Sumatra Caffeine	Aus	Bris	6.33	0.00	0
Ethiopian Sidarmo	USA	Phil	6.57	0.00	0
Kenya Caffeine	USA	Phil	6.85	0.00	0
Ghirardelli Double Chocolate	USA	Phil	8.30	2.80	0
Tanzania Caffeine	USA	Phil	9.11	0.00	0
Expresso Roast	Aus	Bris	8.92	0.00	0
Columbian Caffeine	USA	Phil	8.41	0.00	0
Brazil Ipenema	USA	Phil	9.27	0.00	0
Komodo Dragon	USA	Phil	10.48	0.00	0
Rift Valley	USA	Phil	7.85	0.00	0
Expresso Regalo Caffeine	USA	Phil	8.50	0.00	0
Yukon Caffeine	USA	Phil	8.94	0.00	0
Breakfast Blend	USA	Phil	6.69	0.00	0
Arabian Mocha	USA	Phil	7.06	0.00	0
Decaffeinated Coffees					
Komodo Dragon	USA	Phil	0.23	0.00	0
Komodo Dragon	Aus	Bris	0.26	0.00	0
Sumatra	USA	Phil	0.31	0.00	0
House Blend	USA	Phil	0.49	0.00	0
Breakfast Blend	USA	Phil	0.36	0.00	0
Arabian Mocha	USA	Phil	0.46	0.00	0
Expresso Roast	USA	Phil	0.28	0.00	0
Organic Shade Mexico	USA	Phil	0.32	0.00	0
Verona Blend	USA	Phil	0.32	0.00	0
House Blend	Aus	Bris	0.31	0.00	0

* Abbreviations: USA, United States; Aus, Australia; Phil, Philadelphia; and Bris, Brisbane.

1.0230 ± 0.02 g (n = 20) for the decaffeinated samples, respectively. To each beaker was added 100 mL of drinking fountain water. The sample was placed in a domestic microwave unit (Magic Chef model MCD11E3W) and microwaved at power setting 7 for a preprogrammed time of 3 min. After 3 min, the sample was removed, allowed to cool to room temperature, and the liquid carefully decanted into a clean, dry 200-mL beaker. A fresh portion of drinking water was added to each of the 400-mL beakers. The process of microwaving was repeated. This second portion of liquid was allowed to cool to room temperature before being decanted into each of the previously used 200-mL beakers. To ensure uniformity throughout the analyses, the combined extracts were transferred to a 200-mL graduated measuring cylinder and diluted to 200 mL with drinking water.

To ensure the drinking water was free from any possible xanthines, it was analyzed before and after SPE. A chromatogram of the drinking water prior to SPE is shown in Figure 3.

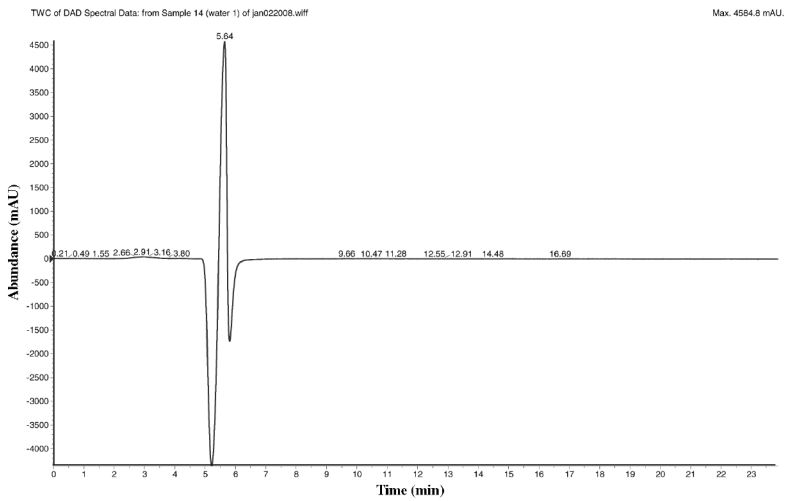

Figure 3. Chromatogram of drinking water prior to SPE.

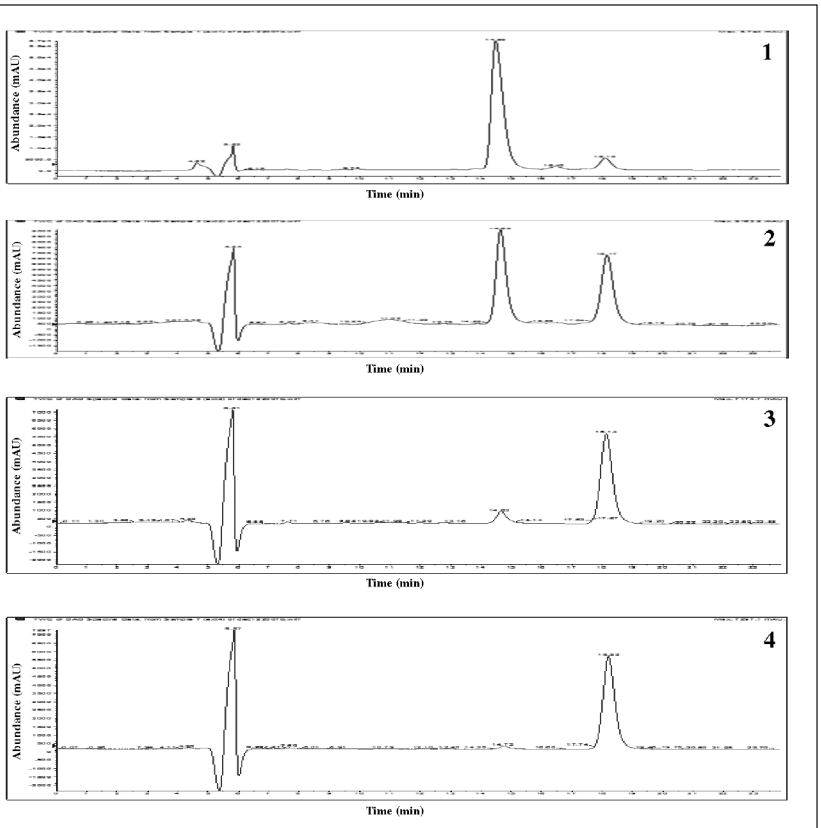

Figure 4. Chromatograms of a caffeinated coffee (1 g) sample undergoing microwave digestion. Steps 1–4 are shown top to bottom. Caffeine Rt = 14.7 min and 8-chlorotheophylline (IS) Rt = 18.4 min.

To determine the number of times the coffee samples needed to be microwaved to fully extract the xanthines, the microwaving and decanting process was performed four times. In this investigation, the liquids containing the micro wave extracts were decanted into four separate 200-mL beakers, and aliquots from each beaker were analyzed by SPE and LC in duplicate. Chromatograms from each of the microwaving steps are shown in Figure 4.

Sample pretreatment: SPE

The pK_a values of caffeine, theobromine, theophylline, and 8-chlorotheophylline were calculated using pKALC software (CompuDrug International, San Francisco, CA) and determined to be 8.30, 7.57, 10.96, and 5.80, respectively. These data would suggest that of the first set of xanthines, only 8-chlorotheophylline had weak acid/neutral characteristics and thus should undergo that particular type extraction, whereas caffeine, theobromine, and theophylline should undergo a basic type of extraction. To test this hypothesis, samples of the xanthines [caffeine, theobromine, theophylline, and 8-chlorotheophylline (100 µL of 1 g/L solutions)] in an aqueous matrix (1 mL) were loaded onto previously conditioned mixed mode SPE columns. To each of the samples was added 1 mL of 0.1 M acetic acid prior to loading.

Column conditioning

The mixed mode columns were conditioned with 1 × 3 mL of methanol, 1 × 3 mL of distilled water, and 1 × 3 mL of 0.1 M acetic acid. These were each allowed to flow through the column under gravity. The height of the aqueous acid was halted just above the column bed to prevent drying out of the columns.

Sample application: aqueous samples and coffee digests

In preliminary studies, caffeine, theobromine, theophylline, and 8-chlorotheophylline were added to 1 mL of 0.1 M aqueous acetic acid at a level of 100 µg to determine

optimum SPE conditions. In subsequent analyses, 8-chlorotheophylline (25 µg) internal standard was added to samples of drinking water/coffee digests (1 mL) in 12-mm × 75-mm test tubes. All samples were analyzed in duplicate. For the calibrators used in the analysis of caffeinated coffee samples, to each aqueous sample was added a known amount of caffeine, theobromine, and theophylline (0, 25, 50, 100, and 200 µg). With decaffeinated coffee samples, the range of xanthine concentrations was 0, 2, 5, and 10 µg. For the preparation of the controls, in addition to the internal standard (25 mg), caffeine was added to aqueous samples at a level of 75 and 150 mg/L for use with caffeinated samples, and a level of 7 mg/L was employed with decaffeinated coffees. Theobromine was added at a level of 7 mg/L. Effectively, three caffeine controls were used to cover the range 1–200 mg/L. To each tube was added an additional 1 mL of 0.1 M of acetic acid. The tubes were vortex mixed for approximately 1 min.

Extraction

The liquids were loaded onto the conditioned sorbent and allowed to pass through with the aid of gravity. After the samples had percolated through the columns, the sorbent was washed with 1 × 3 mL of distilled water; this was followed by 1 × 3 mL of 0.1 M aqueous acetic acid. The columns were then dried under full vacuum for 5 min.

Elution

Elution from the SPE columns was performed using two elution solvent systems. The first elution was carried out using an organic solvent mixture consisting of ethyl acetate/methanol (90:10). The volume of elution solvent employed was 1 × 2 mL. The eluates were collected in glass test tubes (12 × 75 mm) at a rate of approximately 1 mL/min. After collection of the eluates, the tubes were removed from the SPE manifold.

The SPE columns were subsequently washed with 1 × 3 mL of methanol and dried under full vacuum for 5 min. The collection tubes were returned to the SPE manifold in their original positions and the SPE columns were eluted in a second step using a mixture of ethyl acetate/acetonitrile/ammonia (78:20:2). The volume of elution solvent employed was 1 × 3 mL. The elution solvent was collected at a rate of approximately 1 mL/min.

The combined eluates were evaporated to dryness at 37°C using a gentle stream of compressed air in a thermostatically controlled heating block (Reacti Therm III Module, Pierce Chemicals via Fisher Scientific). The residue was dissolved in 1 mL of 0.1% aqueous formic acid and transferred to a 2-mL glass autosampler vial for analysis. Chromatograms of caffeinated and decaffeinated samples are shown in Figures 5 and 6, respectively.

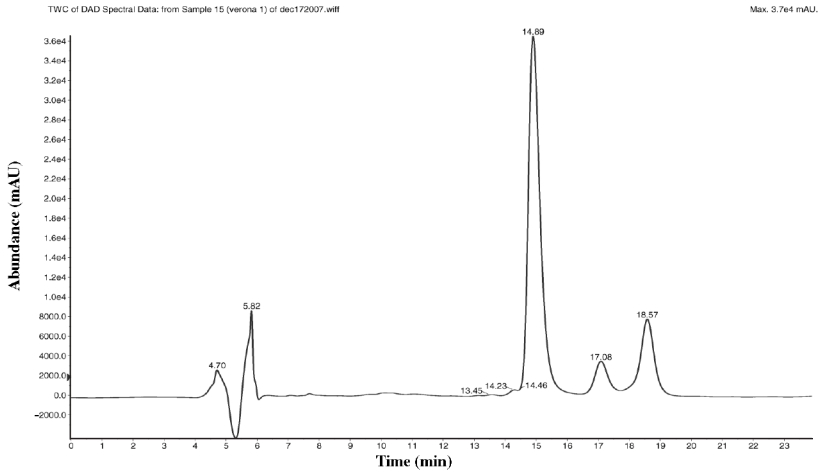

Figure 5. Chromatogram of a caffeinated coffee sample (1 g). Caffeine Rt = 14.7 min and 8-chlorotheophylline (IS) Rt = 18.5 min.

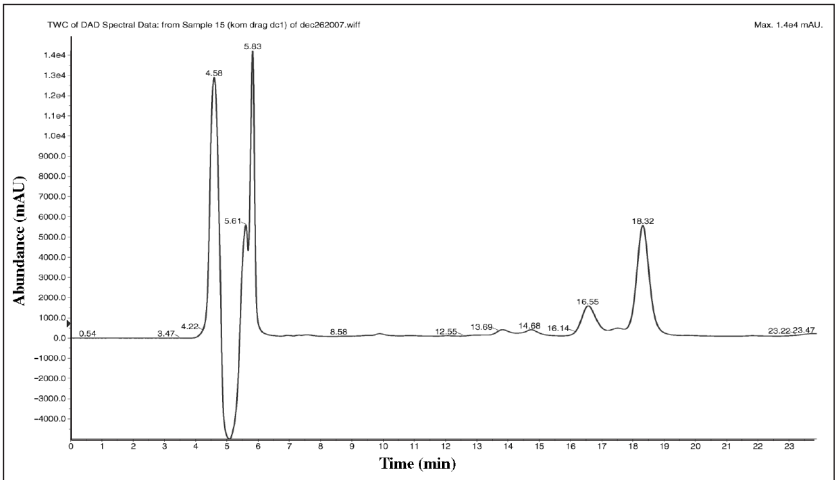

Figure 6. Chromatogram of a decaffeinated coffee sample (1 g). Caffeine Rt = 14.6 min and 8-chlorotheophylline (IS) Rt = 18.3 min.

Results and Discussion

Extraction from mixed mode SPE columns

As previously noted, caffeine, theobromine, and theophylline had predicted basic characteristics with pK_a values of 8.30, 7.57, and 10.96. The other xanthine (8-chlorotheophylline) was predicted to have weak acid/neutral characteristics (pK_a = 5.80).

Caffeine, theobromine, and theophylline were expected to be eluted from mixed mode SPE columns under basic conditions, and 8-chlorotheophylline would be expected to elute under a weak acid/neutral one. When the individual xanthines were applied in aqueous solution of acetic acid (0.1 M) onto mixed mode columns and eluted with ethyl acetate/methanol, it was observed that along with 8-chlorotheophylline, theobromine, theophylline, and caffeine were also detected.

After the SPE columns had been washed with methanol, dried, and eluted with ethyl acetate/acetonitrile/ammonium hydroxide, caf-

feine was detected by LC–DAD. None of the other compounds were detected. The amount of caffeine in this basic extraction was much less than when eluted with ethyl acetate/methanol. Caffeine was detected in basic extract at a ratio of approximately 4:96 with respect to the weak acid/neutral extract.

It is thought by the authors that incomplete ionization of the caffeine, theobromine, and theophylline molecules permitted them to act mainly with weak acid/neutral properties rather than basic ones and would explain why they were eluted mainly under hydrophobic conditions. This hypothesis has not been investigated in this study. Operating the mixed mode SPE column with both modes of elution was observed to collect all of the caffeine, theobromine, theophylline, and internal standard. This was taken as an indication that employing the mixed mode SPE sorbent in both hydrophobic and ion exchange modes was appropriate for the extraction of these types of compounds.

Use of controls and recoveries

As a measure of accuracy and precision of this method, aqueous samples (1 mL) were fortified with the internal standard (25 µg) and known amounts of caffeine (7, 75, and 150 µg). Theophylline was omitted because it was not detected in any of the samples. The aqueous samples were analyzed daily in duplicate over a period of three days. The mean values for the controls were found to be 8 (± 2) µg, 78 (± 3) µg, and 150 (± 3 µg), respectively, for a sample size of n = 6. Because only one sample was found to contain theobromine, the result for the control (7 µg) used in that analysis was found to be 7 (± 2) µg for a sample size of n = 6. Recovery of the xanthines was determined by comparison of unextracted aqueous samples against samples that had undergone solid phase extraction. The mean recoveries were noted as 96 (± 3)% for caffeine, 97 (± 4)% for theobromine, and 96 (± 4)% for theophylline. Limits of detection and quantification were determined empirically by injection of standards into the LC, these were found to be 0.1 and 1.0 mg/L, respectively.

Coffee samples

When a caffeinated coffee sample was extracted by four successive processes of microwaving/decanting, approximately 85% of the total caffeine was extracted in the first step, and approximately 13% of the caffeine was extracted in the second step. The next two steps extracted approximately 1.3% and 0.5%, respectively, of the total caffeine content. Based on this data, coffee samples were subjected to two microwaving/decanting steps prior to SPE and LC.

Concentrations of the xanthines (caffeine and theobromine) in the powdered samples were calculated from individual calibration curves and the results multiplied by a factor of 200 mL/mass (g) of the powdered sample analyzed. Calibration curves were linear over the ranges 1–10 µg for caffeine and 10–200 µg for theobromine.

Of the 32 caffeinated coffee samples analyzed, the range of caffeine concentrations was found to be 5.2 to 12.2 mg/g of coffee powder. None of samples was found to contain theophylline, and only one contained theobromine. This sample of coffee was labeled as a double chocolate type and contained 2.8 mg/g of theobromine and 8.2 mg/g of caffeine. The caffeinated coffee samples show a wide range of concentrations of caffeine even for the same type but obtained in different locations.

The decaffeinated coffee samples (10) contained caffeine at levels 0.28–0.49 mg/g of coffee powder. The mean value was found to be 0.33 mg/g. There was no theobromine or theophylline found in the decaffeinated samples. Concentrations of the xanthine alkaloids in individual samples are shown in Table I.

Based on a conversion factor of 28.34 g/oz (12), our findings suggest that between 147 and 345 mg of caffeine/oz of coffee is available for consumption after microwave extraction of the coffee product. This conversion factor is employed because purveyors of specialty coffees such as Starbucks™ sell their products by the pound (16 oz) and not by the kilogram. The results also show that decaffeinated coffee samples still contain caffeine concentrations around 9.3 mg/oz coffee even after the decaffeination process.

Conclusions

The process of microwave digestion followed by mixed mode SPE and LC is successful method for the determination of total xanthines (caffeine, theobromine, and theophylline) in powdered coffee beans. The information derived from this study should go a long way in helping toxicologists better understand the nature of xanthine concentrations, especially the wide range of caffeine levels to be found in specialty coffees and their relationship to the consumed beverages and levels of these xanthines in biological specimens.

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