LITERATURE CITED
Badger, G. M.; Buttery, R. G.; Kimber, R. W. L.; Lewis, G. E.; Moritz, A. G.; Napier, I. M. *J. Chem. Soc.* **1958**, 2449–2452.
Bruneau, C.; Soyer, N.; Brault, A.; Kerfanto, M. *J. Anal. Appl. Pyrolysis* **1981**, *3*, 71–81.
Crow, W. D.; Paddon-Row, M. N. *Tetrahedron Lett.* **1972**, *31*, 3207–3210.
Gomez, J.; Bruneau, C.; Soyer, N.; Brault, A. *J. Agric. Food. Chem.* **1982**, *30*, 180–182.
Heller, S. R.; Milne, G. W. A. "EPA/NIH Mass Spectral Data Base"; U.S. Government Printing Office: Washington, DC, 1978; NSRDS-NBS 63.
Hurd, C. D.; Macon, A. R.; Simon, J. I.; Levetan, R. V. *J. Am. Chem. Soc.* **1962**, *84*, 4509–4515.
Reichen, W. *Helv. Chim. Acta* **1976**, *59*, 1636–1638.

Jacques Gomez
Christian Bruneau
Noël Soyer
Auguste Brault*
Laboratoires de Chimie Organique et de l'Environnement
de l'Ecole Nationale Supérieure de Chimie de Rennes
35000 Rennes-Beaulieu, France

Received for review October 5, 1981. Accepted April 6, 1982.

Use of Chromatographic Mode Sequencing for Sample Preparation in the Analysis of Caffeine and Theobromine from Beverages

Several bonded-phase sorbents in combination with the effect of several different elution solvents have been evaluated for use in the sample preparation segment of xanthine analyses in beverages. Xanthines are isolated from beverages including coffee, tea, cocoa, and colas in a rapid and highly selective procedure using solid-phase sample preparation, i.e., the use of surface-modified sorbents coupled to the techique of chromatographic mode sequencing. The resultant procedure utilizes Bond Elut columns packed with C_{18} bonded silica (Analytichem International). Average recovery values for the three xanthines ranged from 92% to 98% with coefficients of variation of less than 5% for all three compounds. The caffeine and theobromine levels in several different brands of coffee, tea, and soft drinks were determined by using the described procedure, and the values obtained compared well with those reported by other investigators.

The determination of the levels of caffeine and related xanthines in foodstuffs is becoming increasingly important in the light of recent concern about the health effects of these compounds and their widespread consumption by the public. Several analytical methods exist for the quantitation of caffeine and/or theobromine and theophylline in coffee, tea, soft drinks, and chocolate products. These methods included titrimetry (Horowitz, 1975), spectrophotometry (Grossier, 1978), gas chromatography (Strahl, 1977), and high-pressure liquid chromatography (HPLC) using either normal-phase (Sommadossi et al., 1981) or reversed-phase modes (Kreiser and Martin, 1978). The HPLC methods have the advantages of sensitivity and improved separation, enabling individual quantitation of each of the three xanthines present in a sample. All of the above methods require some degree of sample preparation, usually consisting of a liquid–liquid extraction with a solvent such as benzene or chloroform, followed in some cases by a back-extraction into an aqueous phase to accomplish further separation of caffeine from interfering compounds.

The work described here is an example of solid-phase sample preparation, the use of specifically modified sorbents in combination with chromatographic mode changes to achieve isolation of the compounds of interest from the sample matrix. Several bonded-phase silicas were evaluated in order to determine the best solid phase for this application. Formulation of the mode sequence was based on selection of the appropriate phase together with the evaluation of several solvent systems used to elute the analytes. The objective here was to afford good recovery of the analytes devoid of the naturally occurring chromatographic interferences such as pigments and tannins.

EXPERIMENTAL SECTION

Apparatus. Extraction columns were provided by Analytichem International (Harbor City, CA). Each Bond Elut column contained 100 mg of bonded-phase silica dry packed into a 1-mL polypropylene column. The silica substrate used had a mean particle size of 40 μm and a porosity of 60 Å. The following bonded phases were evaluated: C_{18}, C_8, phenyl, benzene sulfonic acid, carboxylic acid, and cyanopropyl. Extractions were performed by using a Vac Elut 10-place vacuum manifold, also available from Analytichem International.

Analysis was carried out by using a Varian Model 5000 liquid chromatograph equipped with a Varian Vari-chrom variable-wavelength detector (set at 280 mm) and a Linear Instruments Model 255 recorder. The column used contained Sepralyte 10 μm, C_{18} material in a 2.1 mm × 25 cm (narrow bore) configuration (Analytichem International). The column temperature was 55 °C; the mobile phase was acetonitrile–methanol–7 mM H_3PO_4 (4:4:92) at a flow rate of 1.5 mL/min and a pressure of 150 atm.

Reagents. HPLC-grade methanol, acetonitrile, and chloroform were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). All other solvents and inorganic reagents were reagent grade from J. T. Baker (the methanol used for standards and for column washes was HPLC grade). Distilled, deionized water was used to prepare aqueous solutions. Theobromine standard was obtained from Eastman Kodak (Rochester, NY), caffeine from

0021-8561/82/1430-0775$01.25/0

Table I. Recovery of Xanthines from Five-Milliliter Water Samples Extracted with Different Bonded-Phase Silicas[a]

bonded phase	% recovery theobromine	theophylline	caffeine
C_{18}	95	98	93
C_8	66	81	86
phenyl	94	95	93
benzenesulfonic acid	18	22	32
carboxylic acid	24	22	57
cyanopropyl	6	5	8
C_{18}[b]	93	94	98

[a] Using 2 × 500 μL of methanol elution solvent. Average of duplicates. [b] Using 2 × 500 μL of chloroform elution solvent. Average of 30 samples.

Aldrich Chemical Co. (Milwaukee, WI), theophylline from Matheson Coleman and Bell (Cincinnati, OH), and (2-hydroxyethyl)theophylline from Pierce Chemical Co. (Rockford, IL). Aqueous caffeine, theophylline, and theobromine standards were prepared at concentrations of 1.0, 1.0, and 0.5 mg/mL, respectively. Fifty microliters of each of these solutions was used to spike 5-mL water samples for recovery studies. Fifty microliters of (2-hydroxyethyl)theophylline (1 mg/mL in methanol) was used as an external standard.

Coffee and tea samples, purchased locally, were prepared according to package instructions to approximate what would be considered an average concentration level for a cup of coffee or tea. The appropriate amount of dry sample was weighed and then brewed or dissolved in 200 mL of boiling water. After the sample was cooled to room temperature, 250-μL to 5.0-mL aliquots were used for analysis. Dry cocoa was treated in a similar manner. Soft drink samples were extracted directly.

Procedure. Bond Elut columns were inserted into the Vac Elut manifold which was connected to a water aspirator for a source of vacuum. In order to wet the surface of the hydrophobic phases, enabling them to interact with an aqueous sample, we washed each column with 1 mL of methanol followed by 1 mL of water, turning off the vacuum between additions to avoid drying out the sorbent. Samples were then applied and passed through the columns, followed by a 1-mL water wash to rinse off residual sample. (For sample volumes of greater than 1 mL, empty syringe barrels were attached to each column with adapters, to increase the reservoir capacity.) At this point the vacuum was allowed to run for 2–3 min to remove excess water from the columns. Disposable 10 × 75 mm glass tubes were then placed beneath each column in the Vac Elut test tube rack. We added elution solvent in two 500-μL aliquots, turning off the vacuum between additions. The combined eluants for each sample were then evaporated to dryness in a hot water bath and redissolved in 500 μL of mobile phase for injection onto HPLC. Five microliters of each solution was injected at a detector sensitivity of 0.1 AUFS.

RESULTS AND DISCUSSION

Table I shows the recoveries of xanthines obtained with each of the different bonded phases evaluated. On the basis of these results, C_{18} was chosen for further evaluation studies. Various pH conditions and elution solvents were tested for their effect on xanthine recovery. Water samples were adjusted to pH 4 and 7 with 0.1 M sodium phosphate buffers before application to the Bond Elut columns. No significant differences in recovery of any of the three xanthines were observed due to pH; therefore no pH adjustments were recommended for the beverage samples. Solvents tested included methanol, acetonitrile, acetone, 2-propanol, tetrahydrofuran (THF), aqueous THF, ethyl acetate, chloroform, chloroform–acetone (1:1), chloroform–ethyl acetate (1:1), and hexane. Each solvent was also tested on coffee and tea samples for the appearance of the extract (presence of pigments, particulate matter, etc.). Chloroform was chosen for the final procedure based on recovery data, the cleanliness of the extract obtained, and the ease of evaporation.

Table II. Recovery of Caffeine from Water and Decaffeinated Coffee Samples Spiked to Various Concentrations[a]

μg of caffeine added	μg of caffeine recovered from water	% recovery of added caffeine	μg of caffeine recovered from coffee[b]	% recovery of added caffeine
blank	0		10	
25	24	96	33	92
50	50	100	60	100
100	97	97	107	97
250	259	104	270	104
500	498	100	502	98
750	753	100	760	100
1000	968	97	1042	103
2000	1931	97	1840	92

[a] Using C_{18} columns; 2 × 500 μL of chloroform elution solvent. Average of duplicates. [b] The decaffeinated coffee used in this experiment was prepared by dissolving 1.25 g in 200 mL of water.

Table III. Recovery of Xanthines from Replicate Samples Using C_{18}-Bonded Silica Columns[a]

		theobromine	theophylline	caffeine
5-mL water samples[b] (N = 30)	mean:	92.0%	94.4%	98.1%
	SD:	3.9%	4.7%	2.0%
	CV:	4.3%	4.9%	2.0%
1-mL tea samples[c] (N = 10)	mean:	26.5 μg/mL		307 μg/mL
	SD:	0.5 μg/mL		11.9 μg/mL
	CV:	2.0%		3.9%

[a] 2 × 500 μL of chloroform elution solvent. [b] Spiked with 50 μg of caffeine and theophylline and 25 μg of theobromine. [c] 2.35 g of bagged orange pekoe tea brewed for 5 min in 200 mL of hot water.

These results key the following chromatographic mode sequence to achieve sample preparation of xanthines from beverages. (1) The xanthines and interfering substances are retained on a C_{18} surface by passing the beverage through the sorbent. This surface is deactivated so that only the C_{18} moiety is active; i.e., no secondary chromatographic interactions from residual silanols on the silica substrate are present. (2) Selective elution of the xanthines is accomplished by a change of chromatographic mode. Chloroform is passed through the C_{18} sorbent, a drastic change in mobile phase (aqueous to nonpolar), which preferentially elutes the xanthines, leaving the interferences on the C_{18} surface.

Linearity of the method was tested by adding caffeine in increasing amounts (from 25 μg to 2.0 mg) to 1-mL water samples and 1-mL decaffeinated coffee samples. These data, shown in Table II, demonstrate that the capacity of the columns is not affected by the presence of interfering compounds in coffee at these concentrations.

Table III shows the results of precision studies performed with 5-mL spiked water samples and 1-mL tea samples. The water samples were extracted in groups of

Table IV. Xanthine Levels in Beverages Determined by the Bond Elut C_{18}/HPLC Method[a]

	quantity of product used in the preparation of sample	mg of xanthines/sample	values reported by other investigators for similar products
decaffeinated coffee	1.75 g/200 mL	1.9 mg of caffeine/200 mL	1.25-2.25 mg of caffeine/200 mL[b]
instant coffee	1.90 g/200 mL	43.0 mg of caffeine/200 mL	54-76 mg of caffeine/200 mL[b]
ground coffee: weak	5.00 g/200 mL	63.0 mg of caffeine/200 mL	
strong	10.0 g/200 mL	118 mg of caffeine/200 mL	100-150 mg of caffeine/180 mL[c]
black tea	2.64 g/200 mL	4.4 mg of theobromine/200 mL	25-70 mg of caffeine/180 mL[c]
orange pekoe tea	2.35 g/200 mL	5.3 mg of theobromine/200 mL 61 mg of caffeine/200 mL	
instant tea	1.00 g/200 mL	1.8 mg of theobromine/200 mL 29 mg of caffeine/200 mL	29-33 mg of caffeine/200 mL[b]
cocoa	0.1 g/200 mL	24 mg of theobromine/g 3.8 mg of caffeine/g	15-27 mg of theobromine/g[d] 0.8-3.5 mg of caffeine/g[d]
cola	as purchased	37 mg of caffeine/12 oz	40-72 mg of caffeine/12 oz[c]
guarana-based soft drink	as purchased	2.4 mg of caffeine/12 oz	

[a] Average of duplicates. [b] Strahl (1977). [c] Grossier (1978). [d] Zoumas (1980).

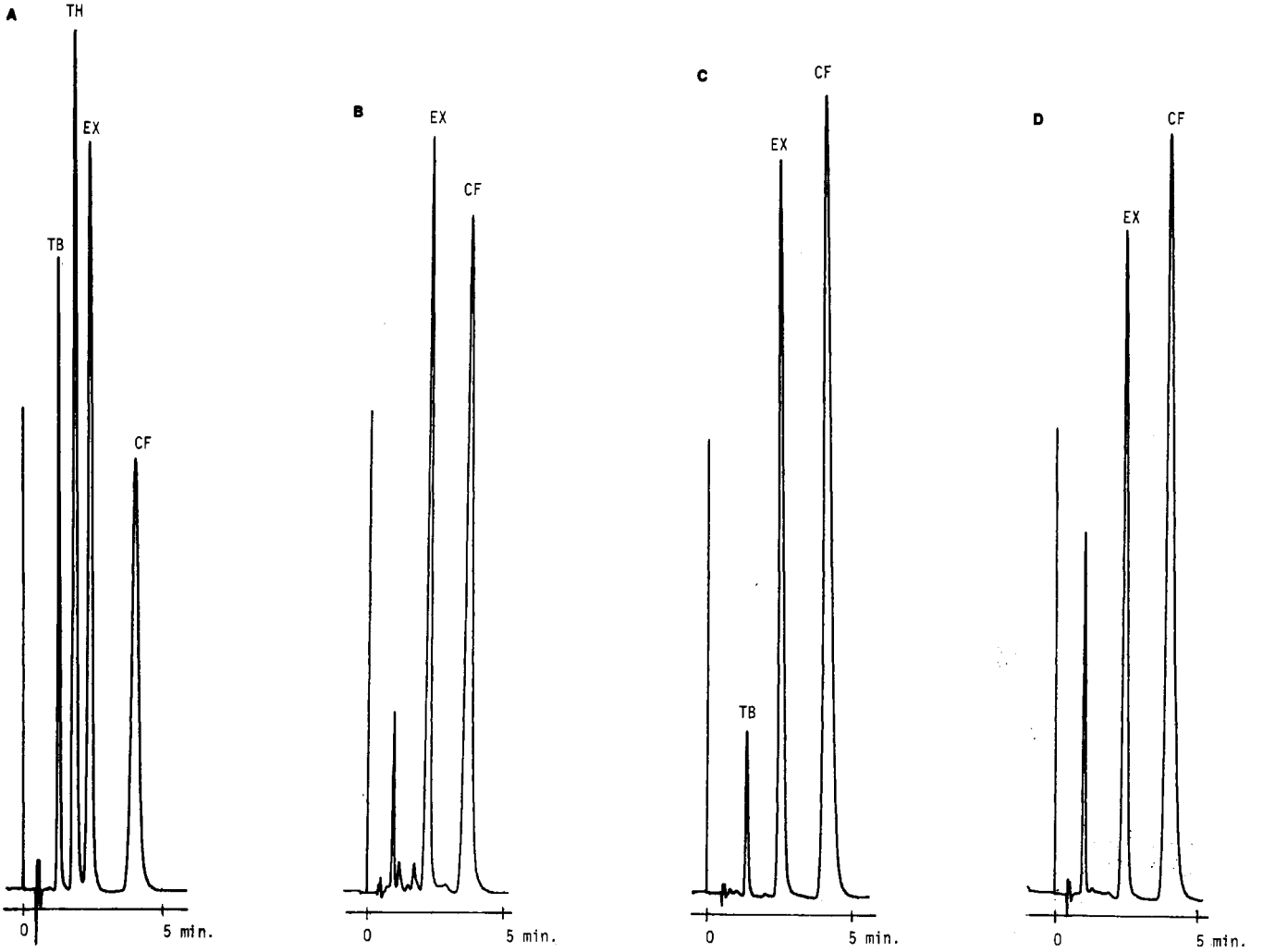

Figure 1. Chromatography of Bond Elut beverage extracts on a Sepralyte 10 μm C_{18}, 2.1 mm × 25 cm HPLC column. Mobile phase = acetonitrile–methanol–7.0 mM H_3PO_4, 4:4:92; flow rate = 1.5 mL/min; temperature = 55 °C; pressure = 150 atm; samples in 500 μL of mobile phase; 5 μL injected at 0.1 AUFS. (A) Standard containing 25 μg of theobromine (TB), 50 μg of theophylline (TH), 50 μg of (2-hydroxyethyl)theophylline external standard (EX), and 50 μg of caffeine (CF). (B) Bond Elut extract of 250 μL of brewed coffee. (C) Bond Elut extract of 500 μL of brewed tea. (D) Bond Elut extract of 1 mL of cola.

10 on three different days with three different lots of columns. Tea samples were run to show the precision is not affected by the presence of other compounds occurring in beverages. Table IV lists the concentrations of xanthines in 10 beverages, quantitated by using the described method. Available reference values are also shown in Table IV for comparison. Figure 1 shows typical chromatograms for a standard and for Bond Elut extracts of coffee, tea, and cola.

CONCLUSION

The application of C_{18}-bonded silica sample preparation columns to the extraction of caffeine and theobromine from beverages allows for rapid determinations with high

reproducibility and linearity over a wide range of xanthine levels. The Bond Elut extraction procedure, when used in conjunction with the modified HPLC procedure presented here, provides for the complete analysis of 10 samples in a total of 60–70 min, from extraction to chromatographic results. This fact, when considered with the simplicity of the method and the selectivity with which xanthines are separated from interfering compounds, makes the method advantageous for any laboratory involved in the routine analysis of caffeine.

LITERATURE CITED

Grossier, D. S. *Am. J. Clin. Nutr.* **1978**, *31*, 1727–1731.
Horowitz, W., Ed. "Methods of Analysis of the Association of Official Analytical Chemists"; AOAC: Washington, DC, 1975; Method 15.022.
Kreiser, W. R.; Martin, R. A. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 1424–1427.
Sommadossi, J. P.; Aubert, C.; Cano, J. P.; Durand, A.; Viola, A. *J. Liq. Chromatogr.* **1981**, *4* (1), 97–107.
Strahl, N. R. *J. Agric. Food Chem.* **1977**, *25* (2), 233–235.
Zoumas, B. L. *J. Food Sci.* **1980**, *45*, 314–316.

Sheryl J. Reid
Thomas J. Good*

Analytichem International, Inc.
Harbor City, California 90710

Received for review August 3, 1981. Accepted April 5, 1982.

Determination of Triforine in Fruit Crops as *N,N'*-Bis(pentafluorobenzoyl)piperazine

A method was developed for the determination of triforine and its piperazine metabolites in fruit. The procedure involves hydrolysis to piperazine, isolation of the piperazine by ion-exchange chromatography, and determination of the pentafluorobenzoyl derivative by gas–liquid chromatography with electron capture detection. The minimum limit of detection was 0.05 ppm, and recoveries from five crops fortified at the 0.10-ppm level averaged 91%.

Triforine [*N,N'*-piperazine-1,4-diylbis[1-(2,2,2-trichloroethyl)formamide] is a systemic fungicide registered for use on several crops. Piperazine and *N*-[2,2,2-trichloro-1-(piperazin-1-yl)ethyl]formamide (TF/2) have been identified as metabolites in barley plants (Rouchaud et al., 1978). Piperazine has also been found to be present in aqueous solutions of triforine after irradiation with UV light (Buchenauer, 1975). One method for the analysis of triforine residues involves extraction with acetone, followed by acid hydrolysis and gas–liquid chromatographic determination of the liberated chloral hydrate (Bourke et al., 1977). While this procedure is satisfactory for the parent compound, TF/2 would be underestimated and piperazine would not be determined. Triforine and its metabolites may be analyzed by three separate determinations performed after chloroform extraction, partitioning with 0.1 N HCl, and hydrolysis of the chloroform extract, aqueous HCl phase, and solid residue (Rouchaud, 1977). The present method was developed to provide a relatively simple procedure capable of determining the total residue of triforine and its metabolites in a single analysis.

MATERIALS AND METHODS

Materials. Cation-exchange resin, Dowex 50 w X8, 100–200 mesh (Sigma Chemical Co., St. Louis, MO), was purified by washing with alkali and acid as described previously (Newsome, 1974). Ion-exchange columns were prepared containing a 3-mL settled volume of resin (Newsome, 1974) and were washed sequentially with 1 N NaOH (15 mL), water until neutral, 1 N HCl (15 mL), and water until neutral.

Silicic acid for adsorption chromatography (Woelm, activity I, 100–200 μm) was purchased from ICN Pharmaceuticals Inc., Cleveland, OH, and was deactivated by the addition of 5% water.

Pentafluorobenzoyl chloride reagent was prepared by dissolving pentafluorobenzoyl chloride (0.1 mL, PCR Research Chemicals, Inc., Gainesville, FL) in 10.0 mL of dichloromethane.

The *N,N'*-bis(pentafluorobenzoyl)piperazine standard was prepared by stirring piperazine (Aldrich Chemical Co., Inc., Milwaukee, WI, 0.4 g, 4.7 mmol) and pentafluorobenzoyl chloride (2.8 g 12.6 mmol) in a solution of acetonitrile (10 mL) and 2 M K_2CO_3 (10 mL) for 1 h. The solid that precipitated during the reaction was removed by filtration, washed with water and then hot acetone, and air-dried to yield 1.2 g (53%) of white powder. The product, mp 293–296 °C, gave a strong molecular ion at m/z 475 on electron impact mass spectrometry and a single peak on gas–liquid chromatography. The reference standard was dissolved in toluene and diluted serially in toluene to give a solution containing 50 pg/μL for gas–liquid chromatography.

Triforine, labeled as 99.6% pure, was obtained from Celamerck, Gmbh Co., Ingelheim/Rhein, West Germany. Solutions used for fortification were prepared in dimethyl sulfoxide and were added to samples in volumes of 0.1 mL.

Analytical Procedure. Samples of homogenized crop (10.0 g) were weighed in 125-mL flasks and refluxed with 1 N HCl (25 mL) for 2 h. The cooled hydrolysate was filtered through Whatman No. 1 paper on a Büchner funnel, and the solids were rinsed with 1 N HCl (15 mL).

The filtrate was transferred to a 1.5 × 20 cm chromatographic tube containing 3 mL of cation-exchange resin and permitted to percolate through it at a flow of approximately 20 mL/h. The column was washed with 1 N NaCl (10 mL) and then piperazine eluted with saturated (35 °C) $NaHCO_3$ (5 mL).

An aliquot of the $NaHCO_3$ eluate (1.0 mL) was placed in a 15-mL centrifuge tube containing 2 M Na_2CO_3 (9 mL)

0021-8561/82/1430-0778$01.25/0