Analysis of Phloxine B and Uranine in Coffee by High-Performance Liquid Chromatography and Capillary Zone Electrophoresis after Solid Phase Extraction Cleanup

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A method was developed for the analysis of phloxine B and uranine, photoactive dyes being evaluated as fruit fly toxicants, in coffee cherries and green and roasted beans. The analytes were measured by high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) using visible and fluorescence detectors after cleanup with disposable amino cartridges. A mixture of methanol (MeOH)/acetonitrile/*n*-butylamine (*n*-BA) (1/1/0.05) effectively extracted phloxine B and uranine from coffee cherries and green beans. The method yielded good recoveries of phloxine B (66−89%) and uranine (75−100%) at spike levels of 0.05−1.0 μg/g from coffee cherries. Good recoveries of phloxine B (82−95%) and uranine (95−110%) were obtained from green beans at spike levels of 0.25−1.0 μg/g. Addition of sodium hexametaphosphate in roasted beans prior to extraction with MeOH/acetone/*n*-BA (1/1/0.05) yielded good recoveries of phloxine B (72−77%) and uranine (79%) at spike levels of 0.5−1.0 μg/g. HPLC and CZE are adequate for determining these analytes. The major advantages of CZE are short analysis time and use of inexpensive columns and aqueous buffer.

Keywords: *CZE; HPLC; solid-phase extraction; phloxine B; uranine; coffee*

INTRODUCTION

Coffee is one of the fastest growing agricultural industries in Hawaii, and the coffee producers compete in world trade on the basis of quality (Goto and Fukunaga, 1991). The high quality of Kona coffee has been known worldwide for over a century, and this is attributed to the coffee cultural practices of the Hawaiian farmers. However, coffee serves as a major host to several Tephritid fruit fly species (i.e., Malaysian, Mediterranean, Melon, and Oriental flies) that were introduced in the islands (Vargas et al., 1995, 1997). Exportation of host plant produce (e.g., papaya and mango) of these flies from Hawaii is subject to quarantine and therefore causes trade limitations of agricultural produce. Tephritid fruit flies also significantly damage crops. Migration of these flies threatens agriculture in fruit fly free areas. Suppression of the fly population can increase crop yield, favor produce trade, and limit migration to a noninfested area.

Currently, malathion is the primary insecticide being used to control these flies, but it has received strong public opposition because of perceived public health and environmental concerns (Service, 1995). SureDye, containing phloxine B and uranine (Figure 1), has shown to effectively control fruit flies and could replace malathion (Liquido et al., 1995a,b; Mangan and Moreno, 1995). When fruit flies consume phloxine B and are exposed to light, phloxine B presumably generates singlet oxygen which is toxic to fruit flies. Uranine is assumed to act as a synergist, although the exact mode of action is yet unknown (Heitz, 1995).

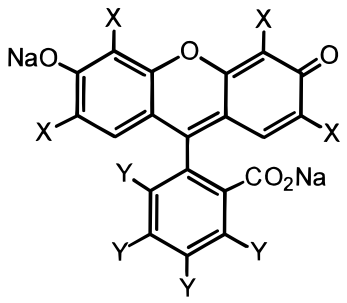

Figure 1. Chemical structures of phloxine B (where X = Br, Y = Cl) and uranine (where X = Y = H).

Phloxine B and uranine are xanthene dyes widely used for drugs and cosmetics in the U. S. Phloxine B and uranine have low mammalian toxicity [phloxine B acute oral LD_{50} (mg/kg) rat 8400, mouse 310, dog >4600; uranine oral LD_{50} (mg/kg) rat 6700, mouse 4700)] (Klaassen, 1973; Lutty, 1978; McDonald et al., 1974; Yankell and Loux, 1977). An allowable intake of phloxine B is 1.25 mg/kg of body weight for human consumption (Food and Drug Administration, 1982). However, little is known on their toxic effects in wildlife and other nontarget organisms in the ecosystems. Related studies on the environmental effects of these dyes are currently being undertaken.

Field pilot tests were conducted in Hawaii, California, and Guatemala on the use of the xanthene dyes for suppression and eradication of fruit flies (Li et al., 1997a; Liquido et al., 1997). SureDye was aerially sprayed on 126 acres of coffee in Kauai, HI, in 1996. Part of the study was to develop analytical methods to assess the fate of these dyes in the environment. We previously reported an analytical method for phloxine B, uranine, and related xanthene dyes in soil using supercritical fluid extraction (Alcantara-Licudine et al.,

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1997). No method was available for the analysis of phloxine B and uranine in coffee. This paper reports an analytical procedure developed for these dyes in coffee cherries and green and roasted beans.

Phloxine B and uranine are photosensitive, nonvolatile, water soluble, and stable in basic solutions. Thus, extraction of these polar aromatic dyes from plant matrices was difficult. A mixture of solvents and salts were found to effectively extract phloxine B and uranine from various coffee matrices. Solid phase extraction (SPE) was explored as a cleanup procedure for coffee extracts. SPE was widely used for cleaning up plant extracts, food, and environmental samples (Hiemstra et al., 1995; Redondo et al., 1996; Schenck et al., 1994). SPE was used to clean up coffee extracts for the analysis of carbohydrates (Prodolliet et al., 1995, 1996) and chlorogenic acids (Bicchi et al., 1995). Availability of commercial SPE columns made this procedure simple and convenient for routine analysis.

Capillary zone electrophoresis (CZE) is increasingly used for determining pesticide residues in grains (Krynitsky and Swineford, 1995) and environmental samples (Brumley, 1995; Wu et al., 1995). A CZE procedure was recently developed for determining phloxine B and uranine in water samples (Li et al., 1997b). In this study, CZE was used and compared with HPLC for the analysis of these analytes in coffee extracts.

MATERIALS AND METHODS

Coffee Samples. Coffee cherries were collected from a coffee field in Kauai, HI. The cherries were processed and roasted following a standard procedure approved for USDA Interregional Research Project No. 4. Fresh coffee cherries were pulped and then fermented for 20−24 h. The fermented cherries were rinsed and dried in an oven at 53 °C for 16 h to yield the green coffee beans. The green beans were roasted at 200 °C until the beans turned dark to produce the roasted beans. Moisture contents were 16.8, 10.7, and 4.8% for coffee cherries and green and roasted beans, respectively.

Fresh coffee cherries and dry ice [1/1 (w/w)] were chopped by a food processor. The sample was transferred to a mason jar, loosely capped, and stored in the freezer overnight to allow complete volatilization of dry ice. The jar was tightly sealed the following day and stored at −25 °C for subsequent analysis. Green or roasted beans were ground in a coffee grinder to pass a 20 mesh sieve. Ground-up samples were transferred to mason jars and stored at 5 °C.

Samples were fortified by addition of phloxine B or uranine solution in MeOH to 25−50 g of fresh coffee cherries or green beans and 12.5 g of roasted beans. The samples sat about 30 min prior to extraction.

Chemicals and Supplies. Phloxine B and uranine were obtained from ICN Biochemicals (Cleveland, OH). Phloxine B was purified as previously reported (Alcantara-Licudine et al., 1997). Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$), boric acid (H_3BO_3), *n*-butylamine (*n*-BA), and optima grade methanol (MeOH), acetonitrile (ACN), acetone, ethyl acetate (EtOAc), and methylene chloride (CH_2Cl_2) were obtained from Fisher Scientific (Pittsburgh, PA). Other reagents used in this study were of analytical or HPLC grade. SPE columns (amino, SAX, cyano, diol, octadecyl, and phenyl) were from either J. T. Baker (Phillipsburg, NJ) or Varian (Walnut Creek, CA).

Optimization of Extraction Procedure for Coffee. *Cherries.* Various solvents (acetone, ACN, H_2O, and MeOH) alone or in mixtures with *n*-BA and salts [NaCl, $(NaPO_3)_6$, Na_4-EDTA, Na_2SO_4, NH_4OAc, and Na_2CO_3] were tested to extract phloxine B and uranine from coffee cherries. Phloxine B and uranine were spiked at 3 μg/g on whole cherries (50 g) in a 500 mL Erlenmeyer flask or on ground-up cherries in a 1 quart mason jar covered with aluminum foil. The spiked samples sat about 30 min and were swirled every 10 min. The whole cherries were extracted with 100 mL of solvent three times by shaking the flask on a Burrel shaker for 10 min. The ground-up cherries were homogenized with 100 mL of solvent with a Sorvall Omni mixer at high speed (4 on a scale of 10) and repeated twice. The extracts were combined and decanted through glasswool to a 1 L round-bottom flask. After the extract was concentrated by rotary evaporation to about 100 mL, it was defatted twice with 50 mL of hexane. The defatted extract was then concentrated to 50 mL. An aliquot of the extract was filtered through a 0.45 μm acrodisc filter for HPLC analysis.

Roasted Beans. Phloxine B and uranine were spiked in roasted beans (12.5 g) at 5 μg/g and extracted three times with 100 mL of MeOH, ACN, acetone, or a mixture of these solvents and 5 g of $(NaPO_3)_6$ or Na_4EDTA. Clarifying agents, e.g., MgO (Dick, 1995) or lead acetate [$Pb(OAc)_2$] (Tsumura et al., 1994) were evaluated for cleaning up extracts prior to SPE. Liquid−liquid partitioning was also tried to eliminate interferences. Water (20 mL) was added in extracts (100 mL) which were then partitioned twice with hexane, EtOAc, CH_2Cl_2, or methyl *tert*-butyl ether (MTBE) (2 × 50 mL).

SPE Cleanup for Coffee Extracts. Several types of SPE columns (amino, SAX, cyano, diol, octadecyl, and phenyl) were preliminarily tested to clean up the extracts. Amino and SAX columns were further investigated. Phloxine B and uranine (10 μg each) in 10 mL of MeOH/ACN/*n*-BA [1/1/0.05 (v/v/v)] were passed through a SPE column (100 mg sorbent) after conditioning with aqueous HCl (1 mL, 0.05 M). After the column was washed with 5 mL of distilled water, the dyes were eluted with NaOH (0.5 mL, 0.5 M)/MeOH (5 mL) from the amino column and with NaOH (0.5 mL, 1 M)/MeOH (5 mL) from the SAX column. Various amounts (5−50 μg) of analytes in 10 mL of MeOH/ACN/*n*-BA (1/1/0.05) were applied on amino and SAX columns (100 mg) to examine column capacity. The efficiency of cleanup and elution was evaluated as recoveries of the dyes from the columns.

Phloxine B and uranine were dissolved in different solvent systems (CH_2Cl_2, ACN, acetone, MeOH, H_2O, or mixtures of these solvents and *n*-BA) at 1 μg/mL, and a 10 mL aliquot of the spike solution was applied on the amino column (100 mg). Column conditioning and elution steps were the same as those described above. Analytes were recovered to evaluate the effect of different solvent systems on the trapping efficiency of the amino column. In a separate experiment, hexane, CH_2Cl_2, EtOAc, ACN, acetone, MeOH, and H_2O (10 mL each) were successively passed through the amino column (200 mg) to examine selective removal of interferences after application of the analytes.

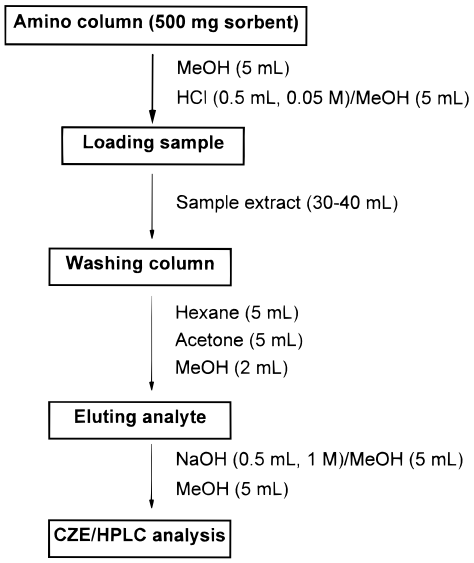
The matrix load was optimized for SPE cleanup of coffee cherry and green and roasted bean extracts. Phloxine B and uranine were spiked at levels of 0.5 and 1 μg/mL in blank coffee extracts (MeOH/ACN/*n*-BA, 1/1/0.05) containing 0.75−10 g equiv coffee matrix. A 10 mL aliquot and a 20 mL aliquot of the 1 μg/mL spike extract were passed through 100 and 200 mg amino columns, respectively. A 40 mL aliquot of the 0.5 μg/mL spike extract was passed through a 500 mg amino column. The dyes were eluted from the column with aqueous NaOH (0.5 mL, 1.0 M)/MeOH (5 mL) followed by an additional 5 mL of MeOH.

Extraction and Cleanup Procedures. *Cherries and Green Beans.* A 25 g sample of coffee cherries or green beans was placed in a 1 pint mason jar and extracted with 100 mL of MeOH/ACN/*n*-BA (1/1/0.05) for 5 min using a Sorvall Omni mixer at low speed (2.5 on a scale of 10). The mixture was filtered through a Buchner funnel, fitted with a no. 7 glass fiber filter, into a 500 mL suction flask. The sample was reextracted twice, and the extracts were combined. The extract was then cleaned up using SPE.

Roasted Beans. The samples (12.5 g) were mixed with $(NaPO_3)_6$ (2.5 g), moistened with distilled H_2O (20 mL), and extracted following the same procedure used for coffee cherries and green beans except for MeOH/acetone/*n*-BA (1/1/0.05) instead of MeOH/ACN/*n*-BA (1/1/0.05).

SPE Cleanup of Coffee Extracts. An optimized SPE cleanup procedure is illustrated in Scheme 1. Sample extracts (30−

Scheme 1. SPE Cleanup Procedure for Phloxine B and Uranine in Coffee Extracts



40 mL) were applied on the amino column (500 mg) after it was washed with MeOH and activated with HCl/MeOH. When the solution was about 5 mm in the column, hexane, acetone, and MeOH were passed successively through the column to remove interferences. Finally, the dyes were eluted with aqueous NaOH/MeOH followed by an additional 5 mL of MeOH. The flow was controlled with a Supelco vacuum manifold (set at 20 psi) connected to a water-suction pump. The elution flow was approximately 5−6 mL/min. After SPE cleanup, the extracts were concentrated to the appropriate volume for HPLC or CZE analysis.

HPLC Analysis. HPLC analysis was done according to the method previously described (Alcantara-Licudine et al., 1997) but with the following modifications: column, Alltima C_{18} LL (Alltech, Deerfield, IL); mobile phase, ACN−0.5 M NH_4OAc buffer with linear gradient increase of ACN 20−80% within 15 min, followed by 100% ACN isocratic elution for 10 min and equilibrated back to 20% ACN for 20 min.

CZE Analysis. The analysis was performed according to an established procedure for phloxine B and uranine (Li et al., 1997b) on a Dionex capillary electrophoresis system (CES-1) (Dionex Corp., Sunnyvale, CA) with ultraviolet−visible (UV−vis) and fluorescence (Fl) detectors. The running buffer was 10 mM $Na_2B_4O_7$ and 50 mM H_3BO_3 (pH 8.5). Buffers and sample solutions were filtered through a 0.45 μm Gelman membrane filter. The fused silica capillary used for separation has an effective length of 65 cm with 75 μm i.d. A new capillary was pretreated by flushing with a 1 N NaOH solution for at least 5 min. Prior to daily analysis, the capillary was conditioned by flushing with distilled deionized water for 3 min, a 0.1 N NaOH solution for 3 min, and finally with the running buffer for 5 min. A constant potential was 20 kV, and the maximum current was 300 μA. Absorptions at 546 and 493 nm were monitored for phloxine B and uranine, respectively. Fluorescent detection of uranine was carried out at 493 (excitation) and 515 nm (emission). The injection mode was by gravity with the sampler head height set at 100 mm for 10 s.

RESULTS AND DISCUSSION

Screening Extraction Solvents (Table 1). Screening various solvent systems suggested that a mixture of MeOH/ACN/*n*-BA (1/1/0.05) is suitable to extract phloxine B and uranine from coffee cherries (Table 1). This solvent system extracted 74 and 83% of the phloxine B and uranine applied, respectively, from whole cherries. The recoveries increased approximately

Table 1. Screening Extraction Methods for Phloxine B and Uranine in Coffee Cherries

		recovery[b] (%)	
solvent system	salt[a]	phloxine B	uranine
H_2O (80 °C)		ND[c](ND)	ND (bp[d])
aqueous NH_4OAc (1.3 M)		ND (ND)	ND (bp)
MeOH		ND (59)	ND (135)
MeOH	Na_2CO_3	38 (19)	80 (81)
ACN		49 (65)	63 (50)
MeOH/ACN (1/1)		79 (70)	81 (72)
MeOH/acetone (1/1)		42 (67)	83 (60)
MeOH/acetone/*n*-BA (1/1/0.05)		59 (58)	63 (60)
MeOH/ACN/*n*-BA (1/1/0.05)		83 (74)	96 (83)
MeOH/ACN/*n*-BA (1/1/0.05)	NaCl	75 (55)	86 (60)
MeOH/ACN/*n*-BA (1/1/0.05)	Na_2SO_4	73 (68)	71 (77)
MeOH/ACN/*n*-BA (1/1/0.05)	$(NaPO_3)_6$	49 (55)	ND (ND)
MeOH/ACN/*n*-BA (1/1/0.05)	Na_4EDTA	47 (60)	56 (50)

[a] 5 g of salt were added. [b] Recoveries were from ground-up or whole (data in parentheses) cherries. Data were the means of two replicates. [c] ND = not detected. [d] bp = broad peak.

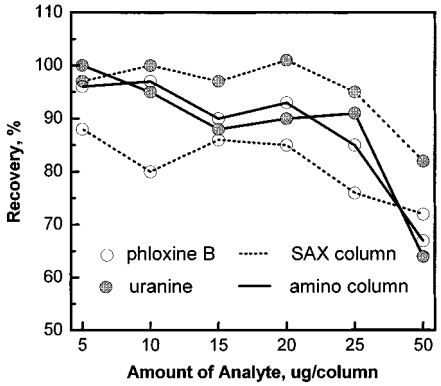


Figure 2. Amino and SAX columns (100 mg sorbent) for trapping phloxine B and uranine.

10% when cherries were homogenized. Addition of salt [i.e., NaCl, Na_2SO_4, $(NaPO_3)_6$, or Na_4EDTA] to the samples did not improve recoveries. Analytes were not extracted by hot water alone or 1.3 M aqueous NH_4OAc even though the dyes are very water soluble. ACN or MeOH alone gave variable recoveries of phloxine B and uranine. Poor recoveries of phloxine B and uranine by MeOH or H_2O may be due partly to sample loss because a thick emulsion was formed during extraction and viscosity of the extract increased during solvent evaporation. Failure to partition phloxine B and uranine in nonpolar solvents such as CH_2Cl_2, diethyl ether, EtOAc, and hexane limited the elimination of interferences by liquid−liquid partitioning. HPLC peak tailing and broadening were encountered after approximately 15−20 injections. The contaminated HPLC column was reconditioned following the method of Dolan and Snyder (1989).

Selection of SPE Columns for Trapping Phloxine B and Uranine (Figure 2). SPE was evaluated for sample cleanup because of its selectivity for a wide range of compounds including polar and anionic analytes. Phloxine B and uranine were quantitatively extracted from water samples using phenyl SPE cartridges (Li et al., 1997b). Among the SPE columns screened (amino, SAX, cyano, diol, octadecyl, and phenyl), the amino and SAX columns effectively retained the analytes when the dyes were dissolved in MeOH/ACN/*n*-BA (1/1/0.05) and passed through the column. The other columns did not effectively retain the dyes. The amino column quantitatively recovered phloxine B (96%)

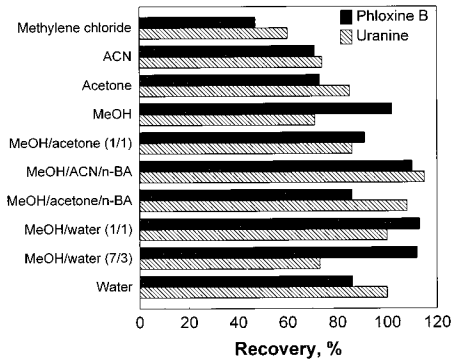


Figure 3. Effect of different solvent systems on the recovery of phloxine B and uranine from the amino column (100 mg sorbent). Ratio of MeOH/ACN or acetone/*n*-BA was 1/1/0.05 (v/v/v).

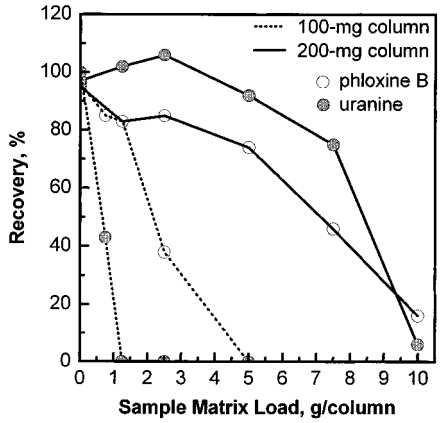


Figure 4. Effect of matrix load on the recovery of phloxine B and uranine from the amino column.

and uranine (100%). The SAX column recovered 77% of the phloxine B and 97% of the uranine applied. The amino and SAX columns effectively trapped anionic phloxine B and uranine and released neutral and cationic interferences such as carbohydrates and chlorophyll.

Subsequent trapping and eluting of the analytes were tested on the amino and SAX columns (Figure 2). The amino column had approximately equal efficiency, illustrated as recovery for phloxine B and uranine. Over 80% of the dyes were recovered when up to 25 μg of uranine and 20 μg of phloxine B were applied per column (100 mg); beyond these loads, recoveries declined. However, the recoveries of phloxine B were lower than uranine on the SAX column. The SAX column is a stronger anion exchanger than the amino column; therefore, a higher concentration of base is required to elute the dyes. The amino column was preferred in this study.

Optimization of Solvent Systems for Trapping Analytes and Effective On-Column Cleanup (Figure 3). In general, the adsorption of the analytes on the amino column varied when phloxine B and uranine were dissolved in different solvents. Both analytes were quantitatively adsorbed (>96%) on the column when the dyes were dissolved in a mixture of MeOH/ACN/*n*-BA (1/1/0.05) or MeOH/H_2O (1/1). MeOH/ACN/*n*-BA (1/1/0.05) also was effective in extracting these dyes from coffee cherries. Therefore, no solvent transfer step was needed for the sample cleanup when the amino column was used. After the dyes were adsorbed on the column, hexane, CH_2Cl_2, EtOAc, ACN, and MeOH (10 mL each) were successively passed through the column. These solvents cleaned up interferences in the columns without removing the target analytes (data not shown). Subsequently, the analytes were eluted with aqueous NaOH/MeOH.

SPE Cleanup for Phloxine B and Uranine Spiked in Coffee Extracts (Figure 4 and Table 2). Phloxine B and uranine were spiked in coffee extracts (MeOH/ACN/*n*-BA, 1/1/0.05) which were equivalent to various amounts of cherries. The amounts of phloxine B and uranine adsorbed decreased as the matrix load increased (Figure 4). The 100 mg column retained over 83% of the phloxine B applied up to 1.25 g equiv matrix load. However, only 40% of the uranine applied, which was first eluted from the column, was recovered with a 0.75 g equiv matrix load. The 200 mg column quantitatively recovered uranine (>92%) when the matrix load was equivalent to 5 g of coffee cherries. Phloxine B recoveries (≥83%) were good up to a 2.5 g equiv matrix load but decreased to 74% at a 5 g equiv matrix load. MeOH insoluble interferences were coeluted with the analytes. The extract formed a two phase solution which was readily separated by centrifugation prior to analysis.

The sorbent/matrix ratio was examined on a larger amino column (500 mg sorbent) (Table 2). A matrix load of up to a 5 g equiv of coffee cherries gave good recoveries (73−103%). The cleanup step yielded a clear, golden yellow extract. Cleanup with this column was also examined for analytes spiked in green and roasted bean extracts. Recoveries were 82−101% up to 2.5 g equiv of green beans but decreased to 46% at a 5.0 g equiv matrix load. When the dyes were spiked in roasted bean extracts, up to a 1.25 g equiv load can be applied without sacrificing good recoveries. However, the roasted bean extract left dark brown colored coextractives in the column which suggested the need of cleanup prior to SPE.

Roasted Bean Extraction (Table 3). When the dyes were spiked in roasted beans, recoveries of phloxine B (32%) and uranine (29%) were low following the same extraction (MeOH/ACN/*n*-BA, 1/1/0.05) and cleanup procedures for coffee cherries or green beans. MeOH/acetone/*n*-BA (1/1/0.05) recovered 35% of phloxine B and 37% of uranine in roasted beans. Roasted coffee differs in chemical composition from raw cherries or green beans by the presence of humic acids and mineral oxides formed during the roasting process (Clarke and Macrae, 1985; Sivetz and Desrosier, 1979). These compounds may strongly interact with the dyes and consequently result in poor recoveries. MgO was commonly used as a clarifying agent in tea analysis (Dick, 1995). MgO turned the dark brown extract to a light yellow brown solution. However, the recoveries of phloxine B and uranine were very low (0−46%) using different combinations of solvents and MgO (Table 3). Addition of lead acetate to clarify the extract as reported by Tsumura et al. (1994) resulted in a clear golden yellow solution; however, the recoveries of phloxine B and uranine were only 57 and 30%, respectively. We previously found that MeOH/*n*-BA/Na_4EDTA were effective modifiers for supercritical fluid extraction and that MeOH/*n*-BA/$(NaPO_3)_6$ was effective for conventional solvent extraction of phloxine B and uranine in soil (Alcantara-Licudine et al., 1997). However, use of MeOH/ACN/*n*-BA/Na_4EDTA yielded low recoveries of phloxine B (9%) and uranine (25%) from roasted beans. Use of MeOH/ACN/*n*-BA/$(NaPO_3)_6$ gave good recovery of uranine

Table 2. Amino Column[a] Cleanup for Phloxine B and Uranine Spiked in Coffee Extracts

equiv sample load (g)	recovery (±SD)[b] (%)					
	coffee cherry		green bean		roasted bean	
	phloxine B	uranine	phloxine B	uranine	phloxine B	uranine
0	96 ± 4	92 ± 7				
0.75	84 ± 6	95 ± 10	96 ± 4	101 ± 6	87 ± 12	90 ± 6
1.25	94 ± 6	103 ± 11	82 ± 9	90 ± 5	88 ± 5	95 ± 3
2.5	89 ± 3	97 ± 3	92 ± 5	92 ± 14	68 ± 3	82 ± 13
5.0	73 ± 11	88 ± 14	46 ± 10	47 ± 19	73 ± 4	69 ± 6
7.5			45 ± 24	17 ± 7		

[a] 500 mg sorbent. [b] Data were the means of three to five replicates. SD = standard deviation.

Table 3. Screening Solvent Extraction Method and Cleanup Prior to SPE for Phloxine B and Uranine in Roasted Beans

solvents	salts	recovery[a] (%)	
		phloxine B	uranine
MeOH/ACN/*n*-BA (1/1/0.05)		31.9	28.5
MeOH/acetone/*n*-BA (1/1/0.05)		34.9	36.7
MeOH	MgO	37.5	46.0
ACN	MgO	ND	ND
MeOH/ACN (1/1)	MgO	19.8	39.1
MeOH/acetone (1/1)	MgO	22.6	17.1
MeOH/acetone (1/1)	$Pb(OAc)_2$	56.7	29.6
MeOH/ACN/*n*-BA (1/1/0.05)	Na_4EDTA	8.9	24.8
MeOH/ACN/*n*-BA (1/1/0.05)	$(NaPO_3)_6$	49.6	79.3
MeOH/acetone/*n*-BA (1/1/0.05)	$(NaPO_3)_6$	70.1	70.4

[a] Data were the means of two to four replicates.

Table 4. Recoveries of Phloxine B and Uranine from Coffee Cherries and Green and Roasted Beans

spike concentration (μg/g)	recovery (±SD)[a] (%)	
	phloxine B	uranine
Coffee Cherries		
1.00	86 ± 3	97 ± 10
0.50	88 ± 7	100 ± 2
0.25	84 ± 7	95 ± 3
0.125	81 ± 8	87 ± 2
0.05	67 ± 2	77 ± 3
Green Beans		
1.0	95 ± 5	110 ± 8
0.5	92 ± 10	105 ± 6
0.25	82 ± 11	95 ± 14
Roasted Beans		
1.0	72 ± 8	79 ± 16
0.5	77 ± 11	79 ± 24
0.25	bp[b]	bp

[a] Data measured by HPLC were the means of three to six replicates. SD = standard deviation. [b] bp = broad peak.

(79%) but not phloxine B (50%). Surprisingly, MeOH/acetone/*n*-BA/$(NaPO_3)_6$ recovered about 70% of phloxine B and uranine from roasted beans. MeOH/acetone/*n*-BA/$(NaPO_3)_6$ also extracted endogenous materials from coffee producing a dark brown solution even after SPE cleanup. Liquid−liquid partition was tested prior to SPE to remove interferences. However, recoveries decreased to 7−47% for phloxine B and 45−69% for uranine after partitioning (without acidification) with CH_2Cl_2, EtOAc, diethyl ether, hexane, or MTBE.

Recoveries of Phloxine B and Uranine Spiked in Coffee Samples (Scheme 1 and Table 4). The analytes in coffee cherries were extracted with MeOH/ACN/*n*-BA (1/1/0.05) and cleaned up with the amino column (Scheme 1). The recoveries of phloxine B and uranine ranged from 67 to 88% and from 77 to 100%, respectively, at 0.05−1.00 μg/g spike levels (Table 4).

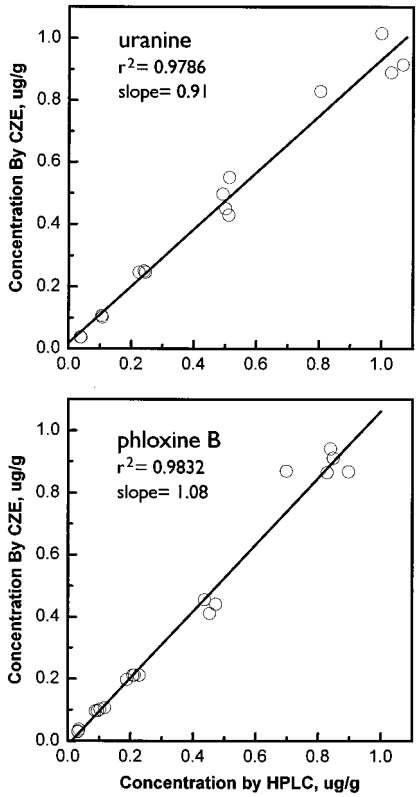


Figure 5. Correlation between HPLC and CZE analyses of uranine and phloxine B in coffee samples.

The extraction and cleanup procedures developed for coffee cherries were applicable to green beans. Good recoveries were obtained for phloxine B (82−95%) and uranine (95−110%) in green beans spiked at 0.25−1.0 μg/g levels. Analyte peak heights slightly decreased and extra peaks appeared on the CZE electropherograms after the extracts were refrigerated overnight at 5 °C. Also, the color of the extracts changed from clear golden yellow to green. This indicated that the analytes in extracts may be unstable in storage. Therefore, quantitation should be conducted immediately after extraction. Extraction using MeOH/acetone/*n*-BA (1/1/0.05) and $(NaPO_3)_6$ gave recoveries of 72−77% for phloxine B and 79% for uranine in roasted beans spiked at 0.5−1.0 μg/g. Recoveries at the 0.25 μg/g spike level could not be determined due to peak broadening.

Comparison of HPLC and CZE Determinations (Figures 5 and 6 and Table 5). Phloxine B and uranine in coffee extracts were determined by CZE and HPLC. The results by CZE correlated closely with those by HPLC (Figure 5). The correlation coefficients (*r*) were about 0.99 for phloxine B (slope, 0.91) and uranine (slope, 1.08).

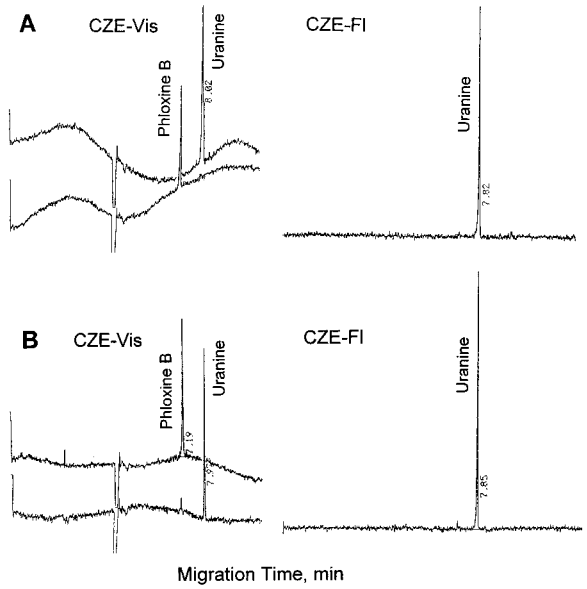


Figure 6. CZE electropherograms of phloxine B [visible (vis), $\lambda = 546$ nm] and uranine [vis; $\lambda = 493$ nm, fluorescence (Fl); $\lambda_{exc} = 493$ nm, $\lambda_{em} = 515$ nm] in coffee cherry extracts (A, top) and in 1.0 μg/mL standard solutions in MeOH (B, bottom).

Table 5. Comparison of HPLC and CZE Analysis of Phloxine B and Uranine

comparison parameter	compound: phloxine B	compound: uranine
Mean Recovery (%) and Coefficient of Variation[a]		
HPLC−vis	81.2 (7.4)	91.4 (5.4)
CZE−vis	81.0 (6.8)	90.6 (8.8)
CZE−Fl		92.4 (5.2)
Limit of Detection (μg/mL)[b] and Coefficient of Variation		
HPLC−vis	0.041 (2.4)	0.071 (2.8)
CZE−vis	0.191 (1.1)	0.125 (2.4)
CZE−Fl		0.075 (2.7)

[a] Data were the means of all recoveries of the analyte spiked at levels of 0.05−1.00 μg/g in ground coffee cherries, and these of all coefficients of variation (CV%, in parentheses). [b] Data were the means of three to five replicates. Signal to noise ratio was approximately 3.

HPLC and CZE gave very comparable average recoveries of phloxine B (81%) and uranine (91−92%) in coffee samples (Table 5). The results by HPLC fluctuated similarly with those by CZE. The mean coefficients of variation (CV%) ranged from 5 to 9% for both HPLC and CZE measurements. The minimum detectable concentrations of phloxine B were 0.04 and 0.19 μg/mL by HPLC−vis and CZE−vis, respectively. The minimum detectable concentrations of uranine were 0.07, 0.13, and 0.08 μg/mL by HPLC−vis, CZE−vis, and CZE−Fl, respectively. The detection limit of phloxine B by CZE−vis was about five times higher than that by HPLC−vis. The detection limit of uranine by CZE−vis was almost doubled compared to HPLC−vis. However, the detection limit of uranine by CZE−Fl was similar to that by HPLC−vis. The high detection limit by CZE was attributed to the small injection volume and short detection path length (i.e., 75 μm i.d. capillary).

Figure 6 shows the CZE electropherograms of phloxine B and uranine in coffee extracts and standard solutions. When an analyte concentration in coffee samples was lower than 0.1 ppm, extracts needed to be concentrated for CZE analysis. Consequently, uranine comigrated with an interference(s) as determined by visible detector. Fluorescence detection is more sensitive and selective, and eliminated interferences. Some difficulties, e.g. peak broadening, changing a guard column, and loss of sensitivity, often occurred during HPLC analysis of coffee extracts, particularly from roasted beans. The CZE method avoided these difficulties. CZE analysis of phloxine B and uranine also had advantages of using aqueous buffer and short analysis time (12 min per run by CZE vs 45 min by HPLC). CZE used a simple and inexpensive column compared to an expensive HPLC column. HPLC and CZE have different separation mechanisms and thus complement each other for chemical identification and characterization. Partitioning of analytes between the stationary and mobile phases and the eluate flow driven by pressure are the main separation mechanism of HPLC. CZE separation is due to differential electrophoretic migration of the analytes and electroosmotic flow of the bulk solution in an electric field.

CONCLUSION

A mixture of MeOH/ACN/*n*-BA (1/1/0.05) adequately extracted phloxine B and uranine from coffee cherries and green beans. MeOH/acetone/*n*-BA (1/1/0.05) and $(NaPO_3)_6$ were used to extract phloxine B and uranine from roasted beans. Amino SPE columns were used for matrix cleanup for the analysis of phloxine B and uranine in all coffee matrices tested. Good recoveries of the dyes were obtained from the coffee samples. HPLC and CZE were comparable for the detection of these dyes in coffee samples. This method will be useful in monitoring phloxine B and uranine residues.

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