Method for the Analysis of Phloxine B, Uranine, and Related Xanthene Dyes in Soil Using Supercritical Fluid Extraction and High-Performance Liquid Chromatography

Jocelyn P. Alcantara-Licudine, Michael K. Kawate, and Qing X. Li*

Department of Environmental Biochemistry, University of Hawaii, Honolulu, Hawaii 96822

The use of supercritical fluid (SF) carbon dioxide (CO_2) modified by organic solvents and inorganic salts or chelating reagents was investigated for the extraction of the xanthene dyes phloxine B and uranine from soil. Methanol (MeOH), *n*-butylamine (*n*-BA), and a chelating agent, ethylenediaminetetraacetic acid tetrasodium salt (Na_4EDTA), were the most effective modifiers of SF CO_2 for quantitative recoveries of phloxine B and uranine in soils with 10−20% moisture at 60 °C/476 atm and 60 °C/272 atm, respectively. At these supercritical fluid extraction (SFE) conditions, recoveries of related xanthene dyes (i.e., 2′,7′-dichlorofluorescein, 4,5,6,7-tetrachlorofluorescein, eosin Y lactone, erythrosin B, and rose bengal) fortified at 25 μg/g in Hawaiian soils ranged from 65 to 93%. Good separation of a mixture of these dyes was achieved by HPLC. A mixture of MeOH, *n*-BA, and sodium hexametaphosphate [$(NaPO_3)_6$] was effective for conventional solvent extraction of phloxine B and uranine from fortified soils. However, SFE was more selective and gave cleaner extracts. Recoveries were comparable to those by solvent extraction.

Keywords: *Phloxine B; uranine; xanthene dye; EDTA; SFE; HPLC; soil*

INTRODUCTION

Phloxine B and uranine are xanthene dyes widely used as coloring additives in drugs and cosmetics. Phloxine B has been used as a food-coloring material in Japan since 1973 (Ito et al., 1994). Demand for determining purity led to numerous studies on its analysis, purification, and synthesis (Gandin et al., 1982; Weisz et al., 1992; Pérez-Ruíz et al., 1994). Phloxine B and uranine also have fluorescent properties useful for biological staining (Lillie, 1969) and optical chemical sensing (Nakagama et al., 1990).

The insecticidal property of xanthene dyes was discovered when mortality, retardation of growth, and a decrease in fecundity of female insects were observed while the dyes were being used as biological markers in an insect ecological study (Yoho et al., 1973). Insect bioassays showed that dye-sensitized photooxidation caused mortality in house flies (Carpenter et al., 1984; Yoho et al., 1976) and other insects (Callaham et al., 1975; Fondren and Heitz, 1978). Recent studies showed that phloxine B and uranine effectively suppressed fruit flies (Liquido et al., 1995a,b). USEPA has approved an experimental use permit to conduct field experiments using these dyes in spray or bait formulations to control fruit flies on coffee in Hawaii, oranges in California, and grapefruit in Texas (USEPA, 1995). Currently, malathion is the chief insecticide to combat fruit flies in Hawaii, but malathion and its oxidative product malaoxon are also toxic to honeybees, lady beetles, and other natural enemies of the pests (Mulla et al., 1981). A more environmentally acceptable fruit fly control agent is needed.

Laboratory toxicity tests indicated that phloxine B and uranine were relatively benign to mammals, although phloxine B was reported to have low toxicity to fish (Tonogai et al., 1979). Assessing environmental effects of phloxine B, uranine, and breakdown products require that reliable analytical methods be established to detect these chemicals in environmental samples. The available methods are intended solely for product purity determination in foods, drugs, and cosmetics (Bell, 1995). These methods are inadequate for analysis of these compounds in complex environmental matrices like soil.

In recent years, supercritical fluid extraction (SFE) has emerged as a valuable technique for rapid and efficient isolation of pesticide residues from environmental samples (Chester et al., 1994; Clement et al., 1995; Sherma, 1995). Carbon dioxide (CO_2) has been the supercritical fluid (SF) of choice, being nontoxic and less expensive and having relatively low critical temperature (32 °C) and pressure (72 atm). SF CO_2, however, being nonpolar does not efficiently extract polar or ionic organic species. Polar SFs such as water or MeOH are possible alternatives for extracting polar compounds (Capriel et al., 1986; Hawthorne et al., 1994). However, the required critical temperatures and pressures for water (374 °C/218 atm) and MeOH (240 °C/78.5 atm) are too extreme for routine application.

SFE of moderately polar analytes like diuron and atrazine from soil using SF CO_2 required the addition of polar solvent modifiers such as MeOH or ethanol (McNally et al., 1988). Good SFE recoveries of methyl eugenol and labile analytes such as naled and cuelure from soil were accomplished (Alcantara-Licudine et al., 1996). Rochete et al. (1993) reported good SFE recoveries of 2,4-dichlorophenoxyacetic acid (2,4-D) from soils with low organic matter by addition of an ionic modifier and derivatization of the free acid to a methyl ester before gas chromatography (GC) determination. Good recovery of 2,4-D was also achieved by *in situ* chemical derivatization and SFE with CO_2 (Hawthorne et al., 1992). 4-Nitrophenol was efficiently extracted from soil by SFE and detected by GC or immunoassay (Wong et al., 1991). In general, advances of SFE for highly polar

* Author to whom correspondence should be addressed [phone, (808) 956-2011; fax, (808) 956-5037; e-mail, qingl@hawaii.edu].

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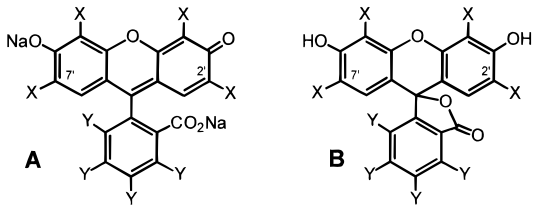

Compound	X	Y
Uranine	H	H
Erythrosin B	I	H
Phloxine B	Br	Cl
Rose bengal	I	Cl

Compound	X	Y
2′,7′-DCF	2′,7′-Cl	H
Eosin Y lactone	Br	H
4,5,6,7-TCF	H	Cl

Figure 1. Structures of seven xanthene dyes (2′,7′-dichlorofluorescein, 2′,7′-DCF; 4,5,6,7-tetrachloroflourescein, 4,5,6,7-TCF).

analytes from environmental matrices have been limited because of poor solubility in SF CO_2, strong matrix−analyte interaction, and practical limitations of using other polar SFs such as water, MeOH, or nitrous oxide (N_2O).

No SFE study on xanthene dyes was found in the literature, although separation of commercial azo, aniline, and anthraquinone dyes by capillary supercritical fluid chromatography was reported (Jackson and Later, 1986). The work presented here describes the systematic development of an SFE procedure for quantitative recovery of phloxine B and uranine from soil. The optimized SFE condition was successfully applied to the extraction of other xanthene dyes including 2′,7′-dichlorofluorescein (2′,7′-DCF), 4,5,6,7-tetrachlorofluorescein (4,5,6,7-TCF), eosin Y lactone, erythrosin B, and rose bengal (Figure 1) from soil. An HPLC method for the separation and quantitation of the seven dyes is described. A solvent extraction method was also developed for quantitative extraction of the dyes from soil.

MATERIALS AND METHODS

Reagents and Standards. Phloxine B and uranine were purchased from ICN Biochemicals (Cleveland, OH). Phloxine B was purified by silica gel column chromatography using acetone/chloroform ($CHCl_3$)/*n*-butylamine (*n*-BA) (90/7/3) as the eluting solvent system. Silica gel (63−200 nm) was obtained from Selecto Scientific (Norcross, GA). Rose bengal, erythrosin B, eosin Y spirit, 2′,7′-DCF, and 4,5,6,7-TCF were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade ammonium acetate (NH_4OAc), *n*-BA, optima grade acetone, acetonitrile (ACN), $CHCl_3$, ethyl acetate (EtOAc), and MeOH were obtained from Fisher Scientific (Pittsburgh, PA). Ethylenediaminetetraacetic acid tetrasodium salt (Na_4EDTA) and sodium hexametaphosphate [$(NaPO_3)_6$] were purchased from Matheson Coleman and Bell (MC/B) (Norwood, OH). EDTA and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium sulfate (Na_2SO_4) was obtained from Mallinckrodt, Inc. (Chesterfield, MO), and other inorganic salts used in this study were purchased from Fisher Scientific. Water for HPLC was filtered through a Sybron/Barnstedt Nanopure II water system set at 18 MΩ cm resistance. CO_2 (99.5% purity) was purchased from Gaspro (Honolulu, HI).

Stock solution (1 mg/mL) of phloxine B was stored in a solution of aqueous NH_4OAc (0.1 M) and MeOH (7/3). Other dyes were stored in MeOH, and all dyes were kept in the dark. Working standards of individual or mixtures of dyes were prepared in MeOH and stored in amber bottles.

Soil and Sand Samples. Three soils, Lihue silty clay, Wahiawa silty clay, and Kaiwiki silty clay loam, were collected from Kauai, Oahu, and Big Island, HI, respectively (Table 1). Soil samples were air-dried, sieved through 20 mesh, and stored in sealed jars at room temperature. Lihue silty clay was used in the SFE optimization study. Soil moisture was adjusted by mixing air-dried soil with appropriate amounts of distilled water at least 1 h prior to use. Oven-dried soil was considered to have 0% moisture. Silica sand was purchased from Merck (Rahway, NJ). Samples (2 g) spiked with appropriate amounts of analyte solution were mixed and allowed to sit in a dark hood for about 1 h before extraction.

SFE Optimization for Phloxine B and Uranine. Samples spiked with dyes were transferred to 2.5-mL stainless steel extraction vessels. The vessels were then placed into an Isco SFX 2-10 extractor (Lincoln, NE) equipped with a microprocessor to control temperature and pressure. A 260 D syringe pump with a circulator maintained at 10 °C was used to pressurize the CO_2. Extraction chamber and restrictor oven temperatures were maintained at 60 and 80 °C, respectively. Extract flow rate was controlled by a stainless steel capillary restrictor (25 cm × 300 μm o.d.) (ISCO) at 5−10 mL/min. A 40-mL test tube covered with foil containing 10 mL of MeOH was used as trapping vial, and about 3−5 bed vol of SF CO_2 was used to extract 2 g of soil. Extractions were performed at 136, 272, 408, or 476 atm. Modifying the solvating power of CO_2 with cosolvents (MeOH, $CHCl_3$, or ACN), adding salts (NaCl, KCl, $NaHCO_3$, $CaCl_2$, $MgSO_4$, Na_2CO_3, Na_2SO_4, Na_4EDTA, EDTA, or EGTA), and/or adjusting soil moisture (0−25%) were tested to optimize SFE recoveries of phloxine B and uranine. Salt modifiers were mixed into the soil, while solvent modifiers were applied on top of the soil before SFE. Data reported are averages of three to six replicates.

SFE Procedure for Xanthene Dye Mixture. Spiked soil (2 g) was thoroughly mixed with 100 mg of Na_4EDTA and quantitatively transferred to a 2.5-mL extraction vessel. After 10 min, 0.05 mL of *n*-BA and 1.0 mL of MeOH were added on top of the soil. The extraction vessel was placed in the extraction chamber for 5 min to equilibrate the temperature and then statically extracted for 5 min with SF CO_2 at 60 °C/272 atm. Dynamic extraction followed, allowing 40 mL of SF CO_2 to pass through the samples at 8−10 mL/min. Samples were re-extracted with SF CO_2 at 60 °C/476 atm after another addition of 1 mL of MeOH. The extracts were collected in the same foil-covered trapping vial containing 10 mL of MeOH. SFE extracts were degassed by sonicating in a lukewarm bath for 0.5 min to remove dissolved CO_2, adjusted volumetrically, and filtered through a Gelman 0.45-μm acrodisc for HPLC analysis.

Solvent Extraction. Several combinations of solvents [$CHCl_3$, ACN, MeOH, or a mixture of $CHCl_3$ and MeOH (1/1)] and salts [Na_4EDTA or $(NaPO_3)_6$] were evaluated before the following procedure was established. Soil samples (2−5 g) were placed into a 90-mL Sorvall steel container, spiked with appropriate amounts of dye solutions and placed in a dark hood for 1 h; 100 mg of $(NaPO_3)_6$ was mixed in 2 g of soil, dispersed in MeOH (40 mL), and basified by adding 0.5 mL of *n*-BA. The mixture was homogenized by a Sorvall Omni mixer at low speed (2.5 on a scale of 10) for 5 min and allowed to settle.

Table 1. Soil Characteristics

soil series	mineralogy	pH[a]	moisture[b] (%)	organic C (%)
Lihue silty clay	clayey, koalinitic, isohyperthermic, tropeptic eutrustox	6.4	5.2	2.6
Wahiawa silty clay	clayey, koalinitic, isothermic, tropeptic eutrustox	6.6	8.4	2.4
Kaiwiki silty clay loam	thixotropic, isothermic, typic hydrandepts	6.2	45.5	5.4

[a] Soil/water ratio = 1/1 (g/mL). [b] Air-dried [(g of water/g of oven-dried soil) × 100]; Kaiwiki series is a unique soil which has a very high water content due to a high content of hydrated X-ray amorphous secondary minerals.

Table 2. Optimization of Pressure and Solvent Modifiers for SFE: Recoveries of Phloxine B and Uranine in Various Solids[a]

			solvent modifier[c]					
analyte[b]	matrix	pressure (atm)	H_2O	MeOH	ACN	$CHCl_3$	MeOH/$CHCl_3$[d]	MeOH/*n*-BA[d]
uranine	Na_2SO_4	136		100 ± 2				
	sand[e]	136	30 ± 1	103 ± 8	31 ± 8	34 ± 1	62 ± 9	
		272	65 ± 20					
	soil[f]	136	ND[g]	59 ± 8				
		272	ND	57 ± 2				
		408	ND	62 ± 6	56 ± 14	13 ± 7	19 ± 1	57 ± 14
phloxine B	Na_2SO_4	136		63 ± 6				
	sand[e]	136	21 ± 1	73 ± 6				
		272	56 ± 13	77 ± 19		75 ± 11	78 ± 20	
	soil[f]	136	ND	9 ± 2				
		272	ND	12 ± 3				
		408	ND	13 ± 2				
		476	ND	23 ± 9	19 ± 8	21 ± 7	5 ± 1	41 ± 20

[a] Data are the average of three to four replicates ± standard deviation (SD). SFE conditions: 60 °C, 2.5-mL extraction cell, and 5 bed vol of CO_2. [b] Spike level was 25 μg/g. [c] Amount of modifiers added on solid matrices: H_2O, 100 μL/g of sand or air-dried soil; organic solvents, 0.25 mL/g of Na_2SO_4 or sand; and 0.5 mL/g of soil which was moistened with H_2O to 20% (w/w) before adding organic solvents. [d] MeOH/$CHCl_3$ and MeOH/*n*-BA ratios were 1/1 and 1/0.05 (v/v), respectively. [e] Silica sand, 0.1−0.5 mm diameter. [f] Air-dried Lihue silty clay. [g] ND = not detected.

The solvent portion was transferred to a 250-mL Teflon tube and the remaining soil re-extracted twice (MeOH, 2 × 40 mL). The combined extracts were centrifuged for 10 min, and the supernatant was decanted into a 250-mL round bottom flask. The MeOH extract was concentrated by evaporation *in vacuo* at 50 °C and adjusted to 10 mL with MeOH. A portion of the extract was filtered through a Gelman 0.45-μm acrodisc filter for HPLC analysis.

HPLC Determination. HPLC analysis was performed using a Perkin Elmer Model 250 binary pump equipped with an Applied Biosystems 10005 diode array detector and a Hewlett Packard 3396 A integrator. Injection volume was 50 μL. HPLC parameters established for the analysis of xanthene dyes were as follows.

A. Phloxine B and uranine: column, Whatman Partisil 5 ODS-3 C_{18} (23.5 cm × 4.6 mm i.d., 5-μm particle size); mobile phase, MeOH and 0.1 M NH_4OAc buffer with linear gradient increase of MeOH from 60 to 100% within 15 min followed by isocratic column wash with 100% MeOH for 5 min and re-equilibrated with 60% MeOH for 10 min at detection wavelengths of 493 and 546 nm for uranine and phloxine B, respectively; flow rate, 1 mL/min; solvent system derived from the method of Weisz et al. (1992) for phloxine B and used in the SFE optimization of phloxine B and uranine.

B. Xanthene dye mixture: several HPLC columns and solvent systems were tested before good resolution of the xanthene dye mixture containing 2′,7′-DCF, uranine, eosin Y lactone, 4,5,6,7-TCF, erythrosin B, phloxine B, and rose bengal was attained. Optimal conditions for good separation of these dyes were as follows: column, Alltima C_{18}-LL 100 A 5U (25 cm × 4.6 mm i.d., 5-μm particle size); mobile phase, ACN and 0.05 M NH_4OAc buffer with gradient elution from 20 to 30% ACN for 10 min, 30−50% ACN for 15 min, and 50−100% ACN for 5 min and re-equilibrated with 20% ACN for 10 min; flow rate, 1 mL/min; detection wavelength, initially set at 493 nm, changed to 525 nm after 9.5 min and 546 nm after 13.5 min; column washed occasionally with ACN/H_2O (1/1) to prevent high-pressure buildup due to buffer salt and sample extractive deposits.

RESULTS AND DISCUSSION

Optimization of SFE Parameters for Phloxine B and Uranine (Table 2). To study the influences of some common SFE parameters on extraction efficiency, sand and Na_2SO_4 were used as solid matrices. Quantitative recovery (100%) of uranine was obtained from Na_2SO_4 using SF CO_2 at 60 °C/136 atm with MeOH as modifier. However, recovery of phloxine B was low (63%) at the same conditions. No detectable levels of these compounds were recovered from Na_2SO_4 or sand using unmodified CO_2 (data not shown). In sand, quantitative recovery (103%) was obtained for uranine at 136 atm using MeOH as a modifier, and moderate recoveries (75−78%) were obtained for phloxine B at 272 atm using CH_3OH, $CHCl_3$, or a mixture of both (1/1) as a modifier. When water was used as a modifier for SF CO_2, recoveries of uranine and phloxine B from sand at 272 atm were 65 and 56%, respectively.

In initial SFE experiments, uranine and phloxine B were poorly extracted from Lihue silty clay soil (data not shown). The recoveries were less than 7% from the air-dried soil using SF CO_2 modified with water, EtOAc, $CHCl_3$, ACN, or MeOH at 60 °C/476 atm (maximum ideal pressure for this extractor).

Addition of MeOH, ACN, or MeOH/*n*-BA (1/0.05) prior to SFE significantly enhanced uranine recoveries (62, 56, and 57%, respectively) at 408 atm after soil moisture was adjusted to 20% (w/w). $CHCl_3$ was a less effective modifier for SF CO_2 to extract uranine from soil. Recoveries of phloxine B in the moistened soil were still low (5−23%) using SF CO_2 modified with ACN, MeOH, $CHCl_3$, or their mixture at 476 atm. When *n*-BA (0.05 mL) and MeOH (1.0 mL) were added, phloxine B recovery almost doubled (from 23 to 41%). Recoveries of phloxine B and uranine increased while increasing pressure and using MeOH as an SF modifier. However, the recoveries of these dyes from soil were still low (≤62%).

Quantitative recoveries of uranine from Na_2SO_4 and sand using SF CO_2 modified with MeOH suggest that MeOH effectively modifies the solvating power of SF CO_2 in desorbing and dissolving the analyte from the matrices (Table 2). Moderate recoveries (63−78%) of phloxine B from Na_2SO_4 and sand using SF CO_2 modified with MeOH or MeOH/$CHCl_3$ indicate that the extracting fluid also dissolves phloxine B but is not adequate to quantitatively remove the analyte from the matrices. SFE recoveries of uranine and phloxine B from soil were lower than those from Na_2SO_4 and sand at the same extraction conditions. Table 2 also showed that the recoveries of phloxine B from the three matrices were lower than those of uranine. These results indicate that phloxine B and uranine strongly interact with soil and that phloxine B is more tightly bound on soil or is less soluble in extracting fluids than uranine.

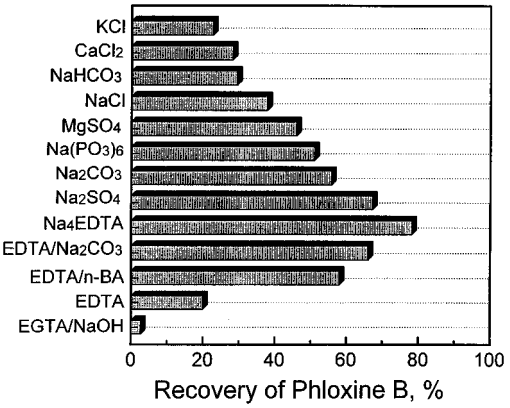

Figure 2. Effect of various salts on recoveries of phloxine B from Lihue silty clay (15% moisture) by SFE. Phloxine B spike level was 25 μg/g. SFE conditions: temperature, 60 °C; pressure, 476 atm; 2.5-mL extraction vessel; 0.5 mL of MeOH/g of soil; 50 mg of salt/g of soil.

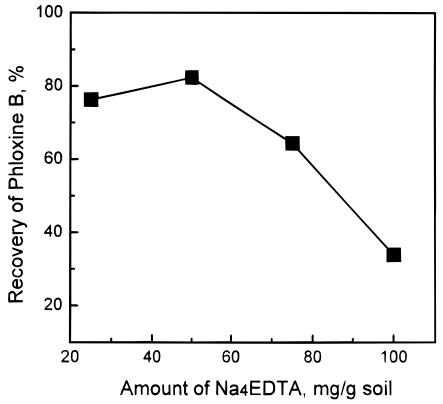

Figure 3. Effect of Na_4EDTA on recoveries of phloxine B from Lihue silty clay (20% moisture) by SFE. Phloxine B spike level was 25 μg/g. SFE conditions: temperature, 60 °C; pressure, 476 atm; 2.5-mL extraction vessel; 0.5 mL of MeOH/g of soil.

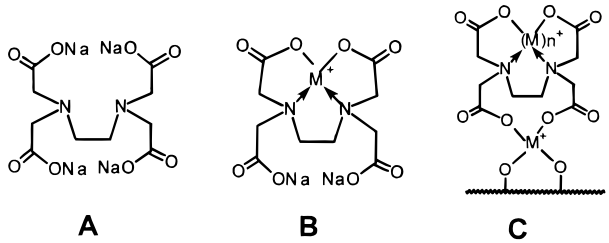

Figure 4. Possible EDTA species as free EDTA anion (e.g., its Na salt) (A), EDTA−metal complex (B), and EDTA−soil particle complex (C) in the presence of SF CO_2 in soil under the experimental conditions; $n = 0$ or 1.

Therefore, further optimization of SFE was focused on interrupting and minimizing analyte−matrix interaction.

SFE of Phloxine B with MeOH and Salts as Modifiers (Figure 2). Hawaiian soils are particularly rich in metallic ions such as Ca, Fe, and Al (USDA Soil Conservation Service, 1972). Soil minerals and organic matter are known to influence the retention of pesticides in soil (Green, 1974). Several inorganic salts and chelating reagents (50 mg/g of soil) combined with MeOH were tested to increase recovery of phloxine B from soil spiked at 25 μg/g (Figure 2). Recoveries with various inorganic salts [KCl, $NaHCO_3$, NaCl, $MgSO_4$, $(NaPO_3)_6$, Na_2CO_3, and Na_2SO_4] ranged from 25 to 67%. Addition of $CaCl_2$ as an ionic modifier improved SFE extraction efficiency of 2,4-D tightly bound on soil (Rochete et al., 1993). However, phloxine B recovery was only 28% with $CaCl_2$. Phloxine B was not recovered using EGTA free acid, and only 3% was recovered when solid EGTA (50 mg/g of soil) and aqueous NaOH (1 N, 100 μL/g of soil) were used. When EDTA free acid was used, phloxine B recovery was only 20%, which may be due to the poor chelating property of EDTA acid form. Coaddition of Na_2CO_3 or *n*-BA yielded phloxine B recovery of 65 or 58%. When Na_4EDTA (50 mg/g of soil) was used, the recoveries dramatically increased from 20 to 78%.

Optimization of Na_4EDTA Levels and Its Hypothetical Mechanism of Enhancing SFE Efficiency (Figures 3 and 4). Na_4EDTA levels were optimized to improve the extraction efficiency of phloxine B in soil (20% moisture). Good recoveries (76−82%) of phloxine B were obtained with 25 or 50 mg of Na_4EDTA/g of soil; however, increasing Na_4EDTA level to 75 or 100 mg decreased recoveries and caused plugging of the extractor outlet (Figure 3). Na_4EDTA should be thoroughly mixed with soil approximately 10 min before adding organic solvent modifiers. When Na_4EDTA was mixed with MeOH, a milky solution formed and deposited white residues on the soil. Resulting recoveries were low (58%).

EDTA and its salts are well-known chelating agents and are used in soil analysis (Cheng, 1990; Crosland et al., 1993). Several chelating agents combined with organic solvents were reported as effective modifiers in SFE of metallic (Wang et al., 1995) and organometallic (Holak, 1995) analytes. The mechanisms of chelating agents in desorbing pesticides from soil particle surfaces in SF CO_2 medium have not been explored, although a chelation reaction is known to occur in SF CO_2 (Jessop et al., 1995).

Possible EDTA forms in soil prior to and/or during SFE include free EDTA anion (e.g., sodium salt), EDTA metal complex, and EDTA complex with soil particles (Figure 4). Na_4EDTA probably interacts stronger with metal than the polar xanthene dyes and thus may exchange and replace the dyes from the adsorbing site. Na_4EDTA may also mask the active sites. MeOH was added to increase the polarity of SF CO_2, thus improving the solubility of these polar dyes in the extracting medium. Ligand formation, ionic displacement, and site covering by Na_4EDTA are suggested mechanisms involved in enhancing extraction efficiency of xanthene dyes from soil.

Effect of Moisture on SFE of Uranine and Phloxine B in Soil (Figure 5). Extraction efficiency of SF CO_2 for phloxine B and uranine was investigated at different soil moisture levels using *n*-BA, Na_4EDTA, or both as modifiers in combination with MeOH. Phloxine B recoveries significantly increased as soil moisture increased to 20% using SF CO_2 modified with MeOH/Na_4EDTA or MeOH/Na_4EDTA/*n*-BA (Figure 5A). At 20% moisture, phloxine B recoveries were similar using Na_4EDTA or Na_4EDTA/*n*-BA; however, recoveries decreased when moisture increased beyond 20%. Similar recovery profiles of uranine, reflecting similar extraction effects, were observed using SF CO_2 modified with MeOH/Na_4EDTA or MeOH/Na_4EDTA/*n*-BA when soil moisture was increased from 0 to 25% (Figure 5B). Maximum uranine recoveries (93−95%) were also obtained at 20% soil moisture. Uranine recoveries slightly increased from 52 to 70% using MeOH/*n*-BA without Na_4EDTA. Other SFE studies also showed that adjusting soil moisture increased recoveries of pesticides (Snyder et al., 1993; Steinheimer et al., 1994).

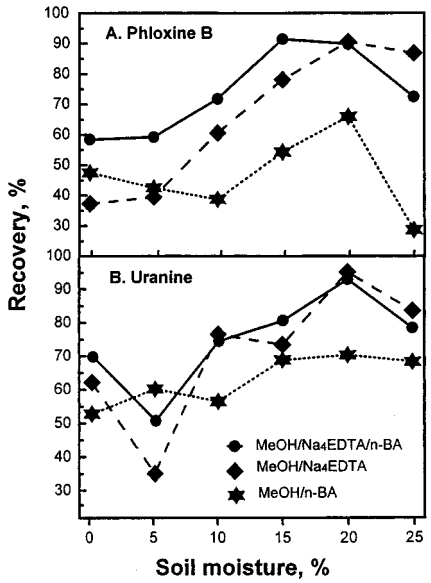

Figure 5. Effect of soil moisture on recoveries of phloxine B (A) and uranine (B) in Lihue silty clay by SFE. Spike level was 25 μg/g. SFE conditions: temperature, 60 °C; pressure, 272−476 atm; 2.5-mL extraction vessel; modifiers, MeOH (0.5 mL/g of soil), *n*-BA (0.05 mL/g of soil), and Na_4EDTA (50 mg/g of soil).

Table 3. Recoveries of Phloxine B and Uranine from Lihue Silty Clay[a] by Solvent Extraction

		recovery[b] (%)	
solvent	salt (50 mg/g of soil)	phloxine B	uranine
$CHCl_3$		ND[c]	ND[c]
ACN		ND	5.5
MeOH		0.3	ND
$CHCl_3$	Na_4EDTA	ND	17.5
MeOH	Na_4EDTA	31.2	43.5
MeOH/$CHCl_3$ (1/1)	Na_4EDTA	15.5	36.5
MeOH + *n*-BA	Na_4EDTA	36.1	34.3
ACN	$(NaPO_3)_6$	ND	2.2
MeOH	$(NaPO_3)_6$	43.4	44.1
MeOH/*n*-BA	$(NaPO_3)_6$	91.4	82.9
MeOH/$CHCl_3$/-*n*-BA (1/1/trace)	$(NaPO_3)_6$	43.0	30.8

[a] Soil moisture was 0.10−0.15 g of water/g of dry soil. [b] Average values of two to three replicates. The spike level was 50 μg of phloxine B or uranine in 5 g of soil. [c] ND = not detected.

When soil moisture was ≥20%, the soil extracts were turbid compared to the clear yellow orange extracts obtained at ≤15% soil moisture. The extractor outlet was frequently plugged, which may be due to coextractives, excess salt, or a reaction product of *n*-BA and CO_2 deposited in the extract collector filter. Excess precipitate was trapped from soil extracts by fitting filter paper or glass fiber at the bottom of the extraction vessel. Although plugging of the extractor filter was minimized, some dyes were adsorbed on the paper filter or glass fiber resulting in decreased recoveries. The extractor should be flushed occasionally with ethanol and the extractant outlet filter frequently changed to avoid plugging problems. The optimum soil moisture ranged from 15 to 20% for SFE of phloxine B and was 20% for uranine using SF CO_2 modified with MeOH/Na_4EDTA/*n*-BA.

Solvent Extraction of Phloxine B and Uranine (Table 3). Various solvents and solvent combinations were quickly screened for extracting phloxine B and uranine from Lihue silty clay soil. The organic solvents alone were not effective in removing phloxine B and uranine from soil. The extraction procedure should use

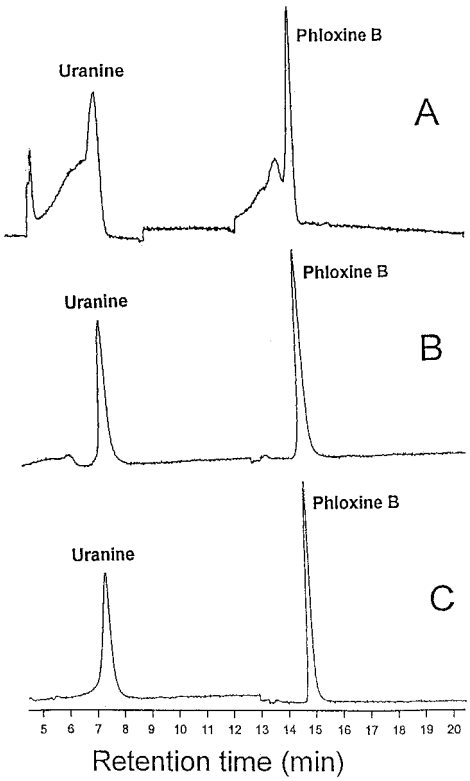

Figure 6. HPLC chromatograms of phloxine B and uranine in a solvent extract after multiple injections (A), SFE extract (B), and standard solution (each 1 μg/mL) (C). HPLC parameters are described in *HPLC Determination* under Materials and Methods.

salt [e.g., Na_4EDTA and $(NaPO_3)_6$], and the mixture should remain basic to extract these dyes from soil. For solvent extraction, Na_4EDTA was not as effective as $(NaPO_3)_6$. Among the combinations of solvents and salts screened (Table 3), MeOH (120 mL) with $(NaPO_3)_6$ (250 mg) and *n*-BA (1 mL) gave the highest recoveries of uranine (83%) and phloxine B (91%) at a fortification level of 25 μg/g. The MeOH extract was very turbid even after centrifugation. This may be due to $(NaPO_3)_6$ which is a well-known soil-dispersing agent. Although filtration of the extract through a 0.45-μm Gelman acrodisc filter produced a clear yellow orange filtrate, tailing and broadening of the uranine and phloxine B peaks were observed after continuous injections of the extract, which are possibly due to accumulation of interferences (Figure 6A).

The above results indicate that SF CO_2 modified by MeOH/*n*-BA/Na_4EDTA has stronger solvating power than the organic solvent MeOH mixed with *n*-BA and Na_4EDTA for extracting phloxine B (92 vs 36% recovery) and uranine (100 vs 34% recovery). The solvent extraction method using MeOH/*n*-BA/$(NaPO_3)_6$ is useful, particularly when a SFE extractor is not available.

Comparison of Phloxine B and Uranine Recoveries from Soil by SFE and Solvent Extraction (Table 4 and Figure 6). Concurrent experiments were conducted to compare and evaluate the two newly developed SFE and solvent extraction methods for phloxine B and uranine in soil. Recoveries of phloxine B and uranine by SFE were comparable with those by solvent extraction. Recoveries of phloxine B from soil spiked at levels of 0.5−25 μg/g ranged from 80 to 92% by SFE and from 89 to 99% by solvent extraction. Uranine recoveries ranged from 91 to 104% by SFE and from 91 to 95% by solvent extraction at the same spike

Table 4. Comparison of Recoveries of Phloxine B and Uranine from Lihue Silty Clay Soil Samples by SFE[a] and Solvent Extraction

	recovery[b] (±SD) (%)			
	phloxine B		uranine	
conctn (μg/g)	SFE	solvent	SFE	solvent
25	92 ± 10	99 ± 10	100 ± 21	95 ± 13
12.5	89 ± 7		100 ± 11	
6.3	87 ± 6	89 ± 5	98 ± 14	
0.75	82 ± 14	94 ± 25	91 ± 11	
0.5	80 ± 5	89 ± 4	104 ± 11	91 ± 6
0.075	69 ± 9		90 ± 1.5	
0.0075	59 ± 10	70 ± 6	57 ± 11	broad peak

[a] SFE conditions: 60 °C, 272 atm for uranine, 476 atm for phloxine B, 2.5-mL extraction cell, and 5 bed vol of CO_2. Modifiers (MeOH/Na_4EDTA/*n*-BA = 1.0 mL/100 mg/0.05 mL) were added on air-dried soil (2 g). [b] Data are the average of four to six replicates.

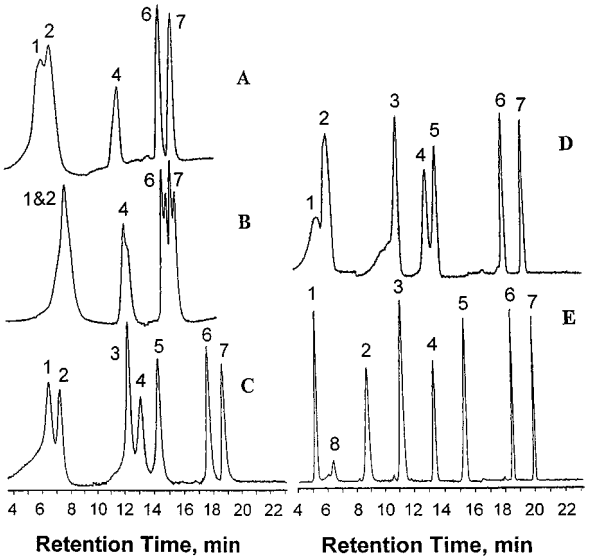

Figure 7. HPLC chromatograms of a mixture of seven xanthene dyes: 2′,7′-DCF (1), uranine (2), eosin Y lactone (3), 4,5,6,7-TCF (4), erythrosin B (5), phloxine B (6), rose bengal (7), and an impurity of 2′7′-DCF (8); injected 50 μL of mixed standard solution of 1−1.5 μg/mL of each dye in MeOH. HPLC parameters are described in the text.

levels. At 7.5 ng/g spike level, phloxine B recovery was 59% by SFE and 70% by solvent extraction. Uranine recovery was 57% by SFE but could not be quantified due to peak broadening by solvent extraction. SFE was more selective and gave cleaner extracts than solvent extraction as shown in Figure 6. The general linear model procedure in SAS indicated that recoveries were similar ($F_{1,49} = 2.20$, $P = 0.14$, $\alpha = 0.05$) between SFE and solvent extraction.

HPLC Analysis of Xanthene Dye Mixture (Figure 7). Several HPLC conditions were considered in separating xanthene dyes in the mixture, and the corresponding chromatograms are shown in Figure 7. Uranine and 2′,7′-DCF was poorly resolved by a C_{18} Nova Pak column (10 cm × 8 mm i.d., 5 μm) for a mixture of five xanthene dyes using MeOH/0.1 M NH_4-OAc at a flow rate of 1 mL/min in gradient elution of 50−80% and 80−100% MeOH for 15 min each (Figure 7A). Addition of triethylamine (TEA; 0.1%) in the eluting solvent initially improved the resolution of uranine and 2′,7′-DCF; however, after prolonged sample injections, peak splitting and overlapping occurred (Figure 7B). The column was reconditioned by reversing the column after washing with organic solvents as recommended by Dolan and Snyder (1989). Erythrosin B, phloxine B, and rose bengal were well resolved but not the other dyes when a Whatman Partisil column (23.5 × 4.6 mm i.d., 5 μm) was tested using MeOH/0.1 M NH_4OAc at 1 mL/min in gradient elution of 60−80% and 80−100% MeOH for 10 and 15 min, respectively (Figure 7C). Under the same flow and gradient rates as for the Whatman Partisil column, an Alltima column (25 cm × 4.6 mm i.d., 5 μm) was also tested, and a poor chromatogram of the seven dyes was produced using MeOH/0.05 M NH_4OAc (Figure 7D). Interestingly, complete resolution of the seven dyes (Figure 7E) was attained with this column using ACN/0.05 M NH_4OAc and adding *n*-BA (0.2%) to the sample solutions with the parameters described in Materials and Methods.

Table 5. SFE Recoveries (%) of Seven Xanthene Dyes Spiked in Hawaiian Soils[a]

xanthene dye	Lihue silty clay	Wahiawa silty clay	Kaiwiki silty clay loam
2′,7′-DCF	88 ± 14	77 ± 12	87 ± 17
uranine	100 ± 3	97 ± 15	85 ± 18
eosin Y lactone	85 ± 2	80 ± 13	88 ± 15
4,5,6,7-TCF	78 ± 2	93 ± 10	65 ± 3
erythrosin B	90 ± 6	92 ± 16	89 ± 13
phloxine B	89 ± 12	88 ± 3	74 ± 7
rose bengal	91 ± 13	86 ± 9	82 ± 8

[a] Data are the average of four replicates ± SD. Spike level was 25 μg/g. SFE conditions: 272−476 atm, 60 °C, 2.5-mL extraction cell, 5 bed vol of CO_2, MeOH (1.0 mL)/Na_4EDTA (100 mg)/*n*-BA (50 μL) modifiers/2 g of air-dried soil.

SFE of Seven Xanthene Dyes in Three Different Hawaiian Soils (Table 5). Seven xanthene dyes were extracted from Hawaiian soils following the SFE conditions optimized for phloxine B and uranine. 2′7′-DCF, eosin Y lactone, and 4,5,6,7-TCF are close analogues of phloxine B and uranine. The iodo-substituted xanthene dyes erythrosin B and rose bengal were included because erythrosin B is a potential fruit fly control agent (Waggoner, 1995) and rose bengal has long been used as a bacteriostatic agent in soil (Lillie, 1969). Three Hawaiian soils of volcanic origin were chosen based on their representative and unique characteristics. The Lihue, Wahiawa, and Kaiwiki soils are slightly acidic with pH values ranging from 6.2 to 6.6 (Table 1). Kaiwiki silty clay loam is of volcanic ash origin and has a capacity to hold a large amount of water. Also, the organic carbon content of this soil is almost 2 times that of Lihue silty clay and Wahiawa silty clay soils.

In Lihue silty clay soil, high recoveries (89−100%) were obtained for phloxine B, rose bengal, erythrosin B, and uranine, but slightly lower recoveries (78−88%) were obtained for 2′,7′-DCF, eosin Y lactone, and 4,5,6,7-TCF. Recoveries (80−97%) of the dyes in Wahiawa soil are comparable to those in the Lihue soil except for a decrease in 2′,7′-DCF (77%) and an increase in 4,5,6,7-TCF (93%) recoveries. Xanthene dye recoveries from the Kaiwiki soil were relatively lower compared to those from the other two soils. Recoveries of phloxine B and uranine from this soil were only 74 and 85%, respectively, and this may be attributed to its higher organic matter and moisture contents. Recoveries of the lactone xanthene dyes 2′,7′-DCF and eosin Y ranged from 87 to 88%. Degradation of 4,5,6,7-TCF in this soil during analysis was observed as evidenced by two breakdown product peaks in the HPLC chromatograms (not shown), resulting in low recovery (65%).

CONCLUSION

Quantitative recoveries of phloxine B and uranine were achieved using SF CO_2 modified with MeOH and *n*-BA in the presence of adequate levels of Na_4EDTA. Using Na_4EDTA-assisted SFE, good recoveries were also obtained for the xanthene dyes 2′,7′-DCF, eosin Y, and 4,5,6,7-TCF which are possible breakdown products of uranine or phloxine B. Good SFE recoveries of the iodo analogues erythrosin B and rose bengal in these soils were also obtained. Excellent separation of these seven xanthene dyes in a mixture was achieved by HPLC.

A solvent extraction procedure for phloxine B and uranine was developed and compared with that of SFE. A combination of MeOH/*n*-BA and addition of $(NaPO_3)_6$ quantitatively extracted these dyes from soil fortified at 25 μg/g. This method will be valuable to laboratories where SFE is not available. These two methods gave comparable recoveries, but SFE was superior in terms of analysis speed, selectivity, and clarity of extracts.

Use of Na_4EDTA in SFE is a promising approach for quantitative recoveries of polar organic analytes from soil. However, considerable optimization is needed before this procedure can be used for general application.

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