Supercritical Fluid Extraction from Soil and HPLC Analysis of Cyanazine Herbicide

Deepa M. Goli, Martin A. Locke,* and Robert M. Zablotowicz

Southern Weed Science Laboratory, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 350, Stoneville, Mississippi 38776

Efficacy of supercritical fluid extraction (SFE) for the recovery of cyanazine (2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropionitrile) from soil was investigated. A reverse-phase HPLC method and a TLC method were developed for quantitative separation of cyanazine from its seven metabolites. Dundee silty clay loam soil treated with ^{14}C-ring-labeled and nonlabeled cyanazine was used. Several SFE parameters were optimized for maximum recovery of cyanazine. Methanol/water (1:1) added directly to the soil matrix was the most efficient modifier. Extraction with supercritical CO_2 at a flow rate of 3.0 mL min^{-1}, a density of 0.90 g mL^{-1}, and an extraction temperature of 50 °C was optimal. With 6 and 20 min static and dynamic extraction times, respectively, cyanazine could be extracted from soil in 40−45 min. With a considerable savings in time and lower waste solvent generation, recoveries (>90%) very similar to conventional extraction procedures could be obtained. Extraction of some of the cyanazine metabolites from soil, under the conditions optimized for cyanazine, was also investigated.

Keywords: *SFE; cyanazine; metabolites; HPLC; TLC*

INTRODUCTION

Cyanazine is a selective systemic herbicide widely used for pre- and postemergence control of most annual grasses and grassy weeds in such crops as corn (*Zea mays*), cotton (*Gossipium hirsutum*), and cereals. In both agricultural and environmental studies, the identification and quantification of residual herbicides and their metabolites is very crucial. Extraction and quantification of pesticides from complex matrices such as soils and sediments require the development of a rapid and precise analytical approach. While there has been remarkable progress in methods involving chromatographic separation and detection, the sample preparation methods can be error-prone and time-consuming. Common methods of extraction, such as multistep liquid−liquid extraction and Soxhlet extraction, can be laborious and time-consuming. In addition, the hazardous nature of many commonly used solvents raises concerns about personnel exposure and the cost and environmental dangers of waste solvent disposal. Supercritical fluid extraction is a valuable alternative sample preparation technique because it minimizes both sample preparation time and solvent disposal volume.

Supercritical fluids have unique solvating properties and have been widely accepted as extraction media (Hawthorne, 1990). In a majority of SFE applications, supercritical CO_2 is employed as the extraction solvent primarily because of its low toxicity and low reactivity and its availability in high purity at low cost. In addition, its low critical temperature (32 °C) and pressure (73 bar) make it a suitable choice because of minimal concerns about thermal decomposition of the analytes. SFE has been used for the extraction of trace organics from many solid matrices, including flavors and fragrances from natural products (Cavley et al., 1994; Huston et al., 1991; Sugiyama and Soato, 1988; Sharma et al., 1991; Hawthorne et al., 1993) and polycyclic compounds and polychlorinated biphenyls from environmental solids (King, 1989; Lee et al., 1992; van-der Velde et al., 1992, 1993). Applications for herbicides in particular include extraction of s-triazines (Janda et al., 1989; Knipe et al., 1992; Rochette and Koskinen, 1996), ureas (McNally and Wheeler, 1988b; Locke, 1993), sulfonylurea herbicides (McNally and Wheeler, 1988a; Howard and Taylor, 1992), chlorophenoxy herbicides (Lopez-Avila and Dodhiwala, 1993), and imazaquin (Reddy and Locke, 1994) from soil, carbamate pesticides from animal tissues (Murugaverl et al., 1993), and several pesticide residues from food samples (Nam and King, 1994; King, 1989; Snyder et al., 1993; King et al., 1993). Although cyanazine has been briefly studied (Janda et al., 1989; Knipe et al., 1992), the emphasis of these studies was not specifically to optimize the extraction of cyanazine from soil. Additionally, previous investigators (for example, Benyon et al., 1972a; Brown et al., 1972; Blumhorst and Weber, 1994) studying chemical and microbial degradation of cyanazine have identified several degradation products/metabolites in soil. Hence it is important to investigate the SFE procedure for these metabolites. The objective of the present study was to optimize supercritical fluid extraction conditions for the extraction of cyanazine and its metabolites from soil and compare the efficacy of this method to conventional extraction procedures.

MATERIALS AND METHODS

Chemicals. Analytical grade cyanazine (**i**) (99% purity) was obtained from Shell Chemical Co. (Houston, TX). Cyanazine metabolites, cyanazine amide (**ii**), 2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanamide; cyanazine hydroxy acid (**iii**), *N*-[6-(ethylamino)-1,4-dihydro-4-oxy-1,3,5-triazin-2-yl]-2-methylalanine; deisopropylatrazine (**iv**), 6-chloro-*N*-ethyl)-1,3,5-triazine-2,4-diamine; and their deethylated metabolites, deethylcyanazine (**v**), 2-[(4-amino-6-chloro-1,3,5-triazin-2-yl)amino]-2-methylpropanenitrile; deethylcyanazine amide (**vi**), 2-[(4-amino-6-chloro-1,3,5-triazin-2-yl)-amino]-2-methylpropanamide; deethylcyanazine hydroxy acid (**vii**), *N*-[6-amino-1,4-dihydro-4-oxy-1,3,5-triazin-2-yl]]-2-methylalanine; and 6-chloro-1,3,5-triazine-2,4-diamine (**viii**), were supplied by E.I. duPont de Nemours & Co. (Wilmington, DE). Ring-labeled [^{14}C]cyanazine (99% purity; specific activity, 0.852 MBq mg^{-1}) was obtained from E.I. duPont de Nemours and Co.

Analytical Procedure. For the HPLC separation and quantification of cyanazine and its metabolites (**i**−**viii**), a reverse-phase column with isocratic methanol/H_2O mobile phase was used. Analysis was carried out by utilizing a Waters Millenium 2010 system (Millipore Corp., Waters Chromatographic Division, Milford, MA) consisting of Waters 600 solvent pump, Waters 486 variable wavelength UV detector, WISP 715 (Waters intelligent sample processor), and Millenium 2010 chromatographic data processor software. A 25 cm × 4.6 mm i.d. Adsorbosphere C_{18} column with 5 mm particle size (Alltech Assoc., Deerfield, IL), 55:45 H_2O/methanol mobile phase at a flow rate of 1.0 mL/ min, and a detector wavelength of 220 nM were determined to be optimum for the analysis. A 10−20 μL injection volume was used. The mobile phase and the standard solutions were prepared from filtered (0.45 μm) HPLC grade solvents.

Thin layer chromatography (TLC) was performed on 20 × 20 cm^2 glass-supported silica gel plates (250 μm) (Whatman LK60F, Whatman Inc., Clifton, NJ). Samples (10−25 μL) were applied with a 20-lane Microspotter (Analytical Instruments, Inc., Baltimore, MD) at 40 °C. The plates were then placed in twin trough chambers (Camag Scientific Inc., Wilmington, NC) with the developing solvent, ethyl acetate/toluene/methanol (50:50:3), and were developed to 10 cm. The chromatographic retention for non-radiolabeled standards was determined using a Shimadzu CS-9000U dual-wavelength flying point scanner (Shimadzu Scientific Instruments, Columbia, MD) with the detector set at 220 nM. The radioactivity distribution in chromatograms was determined with a Bioscan 200 image scanner (Bioscan Inc., Washington, DC).

Soil Preparation. Dundee silty clay loam soil (fine-silty, mixed, thermic, Aeric Ochraqualf) was air-dried (moisture content, 3.5% w/w) and sieved to a uniform 0.5 mm size. For preliminary experiments, 400 g of this soil was treated at a cyanazine concentration of 0.125 μmol g^{-1}. The soil was mixed thoroughly with a spatula, air-dried, ground with a mortar and pestle, and stored in freezer at −20 °C until use. For studies with ^{14}C-radiolabeled cyanazine, 200 g of Dundee soil was treated with analytical grade cyanazine and radiolabeled cyanazine at a final cyanazine concentration of 0.125 μmol g^{-1} (9.50 nCi g^{-1}). The soil was stored at −20 °C until use.

Total Sample Oxidation. ^{14}C-Ring-labeled cyanazine-treated soil (0.300 g) was mixed with 0.30 g of cellulose and oxidized on a Tri-Carb B-306 oxidizer (Packard Instruments, Downers Grove, IL). The $^{14}CO_2$ evolved as a result of sample oxidation was trapped in Carbo-sorb and mixed with Permafluor scintillation cocktail (Packard Instruments, Meriden, CT) for counting on a Packard Tri-Carb 4000 liquid scintillation counter (Packard Instruments). Untreated soil was similarly oxidized for background correction.

Conventional Extraction. Subsamples (3.0 g) of the cyanazine-treated soil were weighed into Corex test tubes with Teflon-lined caps and were extracted with four different solvents: (1) water; (2) methanol; (3) methanol/water (1:1); and (4) methanol/water (4:1) at a ratio of 2.5:1 solvent volume:soil mass. The samples were shaken for 3 h and then centrifuged at 1900*g*. The supernatants were filtered through a 0.45 μm filter (Millipore Corp., Milford, MA) and analyzed by HPLC. Untreated soil was also extracted with methanol/water (1:1) to ascertain the absence of residual cyanazine in the soil before treatment. Soil samples treated with ^{14}C-labeled cyanazine were similarly extracted with methanol/water (1:1) and 1.00 mL of the extract was mixed with Ecolume scintillation cocktail (ICN Research Products, Costa Mesa, CA) for counting.

Supercritical Fluid Extraction. The supercritical fluid extraction of cyanazine was conducted using a Hewlett-Packard Model 7680A supercritical fluid extractor (Hewlett-Packard Co., Avondale, PA). The extractant, CO_2, was subjected to supercritical pressure and temperature conditions prior to entering the extraction chamber containing the soil. A small time period was allowed for equilibration prior to the actual extraction and deposition of the analytes on the solid-phase trap. The fluid CO_2 then passed through a restrictor and was released as gas into the atmosphere. The analytes deposited on the trap were eluted with methanol. The SFE conditions used in this study for cyanazine extraction are listed in Table 1. The extracts containing non-radiolabeled cyanazine were analyzed by HPLC, and the extracts from soil treated with ^{14}C-ring-labeled cyanazine were assayed for radioactivity by scintillation counting. For all experiments, the CO_2 gas was released into a 20 mL vial containing methanol, and the effluent cyanazine was measured by analysis on HPLC or by scintillation counting, whichever was appropriate.

Table 1. Cyanazine Extraction Conditions

Parameters Held Constant	
extractant	SFE grade CO_2
flow rate (mL min^{-1})	3.0
thimble size (mL)	7.5
nozzle temperature (°C)	50
trap temperature (°C)	40
trap	ODS (octadecyl silica)
Parameters Varied	
modifier/volume (mL)	methanol, water, 0.00−0.90
sample size (g)	1.0−6.0
extraction temperature (°C)	40−100
CO_2 fluid density (g mL^{-1})	0.40−0.90
dynamic extraction time (min)	10.0−35.0
static extraction time (min)	3.0−10.0

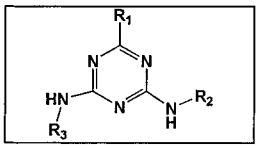
SFE of Cyanazine Metabolites. Metabolites of cyanazine (**ii**−**vi**), 100 μL samples (100 μg mL^{-1} in aqueous methanol) were individually applied to Kimwipe tissue paper [Kimberly-Clark Corp., Roswell, GA], placed in the extraction thimble. The recoveries from this inert matrix by SFE were determined using the conditions optimum for cyanazine extraction. The extracts were analyzed by HPLC. The residual amounts of the metabolites present on the Kimwipe tissue paper were further extracted with methanol/water (1:1) solvent, and these extracts were also analyzed by HPLC.

Soil Incubation Studies. Six subsamples (10.0 g each) of the ^{14}C-radiolabeled cyanazine-treated soil were weighed out in 20 mL beakers. A 2.0 mL of 1% glucose solution (to stimulate microbial activity) and an additional amount of deionized water were added to each beaker to a final moisture content of 32%. The beakers were then covered with parafilm and incubated in the dark at 25 °C. Progress of cyanazine degradation was monitored by extraction of subsamples with 1:1 methanol/water and analysis of the extracts by TLC (above procedure). The identification of radiolabeled metabolites was performed by comparison of the R_f values with those of the standards. Incubation was stopped at 35 days, and the samples were frozen until use.

Subsamples containing 3.0 g of soil were extracted by either the conventional or SFE procedure, and the spent samples from SFE were further extracted by conventional procedure. The conventional extraction procedure consisted of extraction with 2.5:1 (v/w) of methanol/water (1:1) solvent (see above). The extracts from SFE were split for total radioactivity measurement by scintillation counting and for analysis by HPLC and TLC. The extracts from conventional procedure were also analyzed by HPLC, TLC (the extracts were evaporated to dryness and reconstituted in 1.0 mL of methanol), and scintillation counting.

RESULTS AND DISCUSSION

The chemical and microbial degradation of cyanazine has been studied in detail by previous investigators (Benyon, 1972; Benyon et al., 1972a,b; Brown et al., 1972; Blumhorst and Weber, 1994). Several degradation products/metabolites have been identified, although their relative abundance depended on the soil type, soil pH, and several other factors (Blumhorst and Weber, 1994). Cyanazine, its amide, cyanazine acid, and cyanazine hydroxy acid are reported to be major components in soil. Detection of dealkylated components (**iv**−**viii**) in trace amounts in soil and in corn plants (Benyon et al., 1972a) has been reported. Gas chromatographic



Compound #	Name	R_1	R_2	R_3
i	cyanazine	Cl	$C(CH_3)_2CN$	C_2H_5
ii	cyanazine amide	Cl	$C(CH_3)_2CONH_2$	C_2H_5
iii	cyanazine hydroxy acid	OH	$C(CH_3)_2COOH$	C_2H_5
iv	deisopropylatrazine	Cl	H	C_2H_5
v	deethylcyanazine	Cl	$C(CH_3)_2CN$	H
vi	deethylcyanazine amide	Cl	$C(CH_3)_2CONH_2$	H
vii	deethylcyanazine hydroxy acid	OH	$C(CH_3)_2COOH$	H
viii	triazine diamine metabolite	Cl	H	H

Figure 1. Cyanazine and its metabolites.

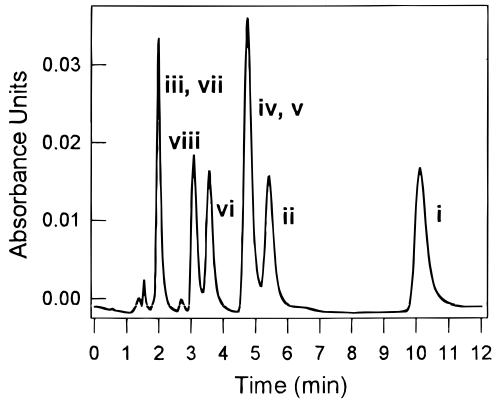


Figure 2. HPLC chromatogram of a composite standard solution (5 mg mL^{-1}) of cyanazine and its metabolites (UV detection, 220 nM). Peak numbers correspond to the compound numbers under Materials and Methods. Chromatographic conditions are reported under Materials and Methods.

methods (for example, Sirons et al., 1973; Muir and Baker, 1978; Thurman et al., 1990) and TLC procedures (for example, Benyon et al., 1972a; Blumhorst and Weber, 1994) have been generally used for analysis of cyanazine and its metabolites. Corcia and Marchetti (1992) have reported a HPLC method for multiresidue analysis, of which cyanazine was one of the components. However, there is no report of an HPLC method for analysis of cyanazine and its metabolites. In this study, we report chromatographic (HPLC and improved TLC) methods for analysis of cyanazine and the above metabolites.

HPLC Analysis. Figure 2 shows the chromatographic separation of cyanazine and its metabolites (**i**−**viii**) with an isocratic elution with 55:45 water/methanol mobile phase at a flow rate of 1.0 mL min^{-1} and UV detection at 220 nM. Elution times were (**i**) 10.1, (**ii**) 5.42, (**iii**) 1.98, (**iv**) 4.73, (**v**) 4.75, (**vi**) 3.55, (**vii**) 1.98, and (**viii**) 3.08 min. For compounds **i**−**vi**, the detection limits were in the range 20−50 ng mL^{-1}. Linearity was verified with standard solutions in the range 50 ng mL^{-1} to 200 μg mL^{-1}. The more polar hydroxy metabolites (**iii** and **vii**) were eluted close to the mobile-phase solvent front. Adjusting the pH of the aqueous component of the mobile phase to 3.40 resulted in better retention of these two components (retention times of 9.1 and 9.4 min for **iii** and **vii**, respectively) without significantly affecting the elution of the other components.

TLC of Cyanazine and Its Metabolites. The R_f values for cyanazine and its metabolites (**i**−**viii**) with ethyl acetate/toluene/methanol (50:50:3) solvent system were (**i**) cyanazine, 0.70, (**ii**) 0.11, (**iii**) 0.00, (**iv**) 0.41, (**v**) 0.50, (**vi**) 0.06, (**vii**) 0.01, and (**viii**) 0.19. Incorporation of methanol improved the separation and the R_f values in general, compared to those reported by Blumhorst and Weber (1994) using only ethyl acetate/toluene system. The R_f values of the hydroxy metabolites (almost 0.0 under above conditions) were improved (approximately, 0.40) using a butanone/H_2O/acetic acid (10:1:1) solvent system, but the separation of **iii** and **vii** was not successful.

Table 2. Percent Recoveries of Cyanazine from Dundee Silty Clay Loam Soil As Affected by Methanol:Water Ratio in the Extraction Solvent

	cyanazine recovery	
extraction solvent	% of applied[a]	SD[b]
methanol	84.5[b]	0.71
water	55.4[c]	0.89
methanol/water (1:1)	92.4[a]	0.48
methanol/water (8:2)	92.0[a]	0.24
methanol/water(1:1)[c]	93.0[a]	1.14

[a] Average from at least three replicate extractions; means followed by the same letter are not significantly different (α = 0.05). [b] Standard deviation. [c] Soil fortified with ^{14}C-ring-labeled cyanazine; concentrations determined by scintillation counting.

Table 3. Effect of Modifier[a] Volume on Cyanazine Recovery from Soil by SFE[b]

	% recovery	
modifier volume (mL)	mean[c]	SD[d]
none	31.8[b]	4.05
0.3	81.8[a]	8.93
0.6	88.5[a]	5.53
0.9	78.4[a]	6.95

[a] Methanol/water (1:1). [b] SFE conditions: 3.0 g of subsample, 0.80 g mL^{-1} CO_2 density, 70 °C extraction temperature, 6.0 and 25.0 min static and dynamic extraction times, respectively. [c] Means (of three replications) followed by the same number are not significantly different (α = 0.05). [d] Standard deviation.

Conventional Extraction. Methanol and aqueous methanol have been reported to be very effective for extraction of triazines from soil (e.g., Cotterill, 1980; Locke et al., 1990). Soxhlet extraction with methanol/water (1:1) (Benyon, 1972; Blumhorst and Weber, 1992) and extraction with acetonitrile/water (8:2) (Sirons et al., 1973) have been used for cyanazine extraction from soil. In the present study, extraction with methanol/water (1:1) and methanol/water (4:1) mixed solvent systems gave greater than 90% recoveries for cyanazine (Table 2). The values were slightly higher than recoveries with methanol alone while water alone gave very poor recoveries (Table 2).

Supercritical Fluid Extraction. Cyanazine was almost quantitatively extracted from an inert matrix (50 μL of methanolic solution applied directly to Kimwipe tissue paper). A set of four extractions (CO_2 fluid density, 0.80 g mL^{-1}; extraction temperature, 70 °C; static and dynamic extraction times of 6.0 and 25 min, respectively) were performed, and the recoveries were very reproducible and quantitative (95.2% with a RSD of 1.2%). Extractions from soil samples and variation of several parameters for optimum extraction efficiency are discussed below.

Modifier. Our initial experiments with soil indicated that the extractability of cyanazine was very poor without any modifier (Table 3). While the addition of either H_2O or methanol alone slightly improved the extraction (results not shown), the modifier effect was

Table 4. Effect of Sample Mass on Cyanazine Recovery from Soil by SFE[a]

sample mass (g)	% recovery mean[b]	% recovery SD[c]
1.0	92.0[a]	0.90
3.0	88.2[a]	1.46
6.0	73.4[b]	5.98

[a] SFE conditions: methanol/water (1:1), 20% (v/w) modifier, 0.80 g mL^{-1} CO_2 density, 70 °C extraction temperature, 6.0 and 25.0 min static and dynamic extraction times, respectively. [b] Means (three replicates) followed by the same letter are not significantly different ($\alpha = 0.05$). [c] Standard deviation.

Table 5. Effect of Extraction Temperature on Cyanazine[a] Recovery by SFE[b]

extraction temperature[c] (°C)	% recovery mean[d]	% recovery SD[e]
40 (3)	90.2[a]	0.37
50 (5)	90.2[a]	1.73
70 (4)	89.3[a]	4.59
80 (4)	79.6[b]	3.00
100 (5)	54.6[c]	6.27

[a] Nonlabeled or ^{14}C-ring-labeled cyanazine-treated soil samples, extracts analyzed by HPLC or by scintillation counting. [b] SFE conditions: 3.0 g of subsample, 0.60 mL of methanol/water (1:1) modifier, 0.80 g mL^{-1} CO_2 density, 6.0 and 25.0 min static and dynamic extraction times, respectively. [c] Numbers in the parentheses following the temperature indicate the number of replications. [d] Means followed by the same letters are not significantly different ($\alpha = 0.05$). [e] Standard deviation.

most pronounced with a methanol/water (1:1) mixed solvent. Further variation in the amount of modifier indicated that 20% (v/w) gave the best results (Table 3). Polar solvents have been added to the sample for improvement in the extractability of some polar herbicides from soil matrices (McNally and Wheeler, 1988a; Knipe et al., 1992; Locke, 1993; Rochette and Koskinen, 1996). Although it is unclear how the modifier facilitates the extractability (analyte solubility or matrix modification), the addition of the modifier directly to the sample matrix has been proved to be more effective than its addition to the extraction fluid (Knipe et al., 1992). Locke (1993) suggested that part of the improvement was due to increased surface area available for extraction after expansion of the clay/humic material by hydration.

Sample Mass. Because of the size (7.5 mL) of the extraction thimble available with the present instrument, the amount of soil that could be extracted was limited. Increasing the sample size from 3.0 to 6.0 g resulted in decreased recovery of cyanazine (Table 4) with increasing variance among replications. This may be due to limitations on the interaction between the supercritical fluid and the matrix because a higher sample size involves a greater surface area for extraction (Locke, 1993). Although a sample size of 1.0 g resulted in slightly higher recoveries, to reduce the sampling error, a sample size of 3.0 g was considered more appropriate for routine extractions.

Extraction Temperature. Variation of the extraction temperature between 40 and 70 °C showed no significant effect on the extraction of cyanazine (Table 5). Further increases in temperature up to 100 °C lowered recovery of cyanazine in the extracts. While thermal degradation was considered as a cause for this decline, an analysis of the extracts by HPLC did not indicate the presence of any degradation products. Detailed mass-balance studies were not conducted, but further investigation indicated that unusually high amounts of cyanazine were collected in the methanol trap for the outlet CO_2 when higher extraction temperatures were used. It was apparent that the lower efficiencies for cyanazine recovery at higher temperatures were due to the loss of cyanazine with the CO_2 gas.

Table 6. Effect of CO_2 Fluid Density on Cyanazine[a] Recovery by SFE[b]

CO_2 fluid density (g mL^{-1})	% recovery mean[c]	% recovery SD[d]
0.40	84.5[c]	1.92
0.60	89.3[b]	0.34
0.80	91.5[a]	0.53
0.90	92.8[a]	0.79

[a] ^{14}C-Ring-labeled cyanazine-treated soil samples, extracts analyzed by scintillation counting. [b] SFE conditions: 3.0 g of subsample, 0.60 mL of methanol/water (1:1) modifier, 50 °C extraction temperature, 6.0 and 25.0 min static and dynamic extraction times, respectively. [c] Means (four replicates) followed by the same letters are not significantly different ($\alpha = 0.05$). [d] Standard deviation.

Table 7. Effect of Static and Dynamic Extraction Time on Cyanazine Recovery from Soil by SFE[a]

extraction time (min) static	extraction time (min) dynamic	% recovery mean[b]	% recovery SD[c]
3.0	20.0	90.9[a]	2.05
6.0	20.0	91.8[a]	1.05
10.0	20.0	92.0[a]	1.30
6.0	10.0	83.7[z]	1.31
6.0	15.0	89.7[y]	2.65
6.0	20.0	91.9[xy]	0.90
6.0	25.0	91.7[xy]	0.43
6.0	35.0	92.2[x]	0.77

[a] SFE conditions: 3.0 g of subsample, 0.60 mL of methanol/water (1:1) modifier, 0.90 g mL^{-1} CO_2 density, 50 °C extraction temperature. [b] Means (four replicates) followed by the same letters are not significantly different ($\alpha = 0.05$). [c] Standard deviation.

CO_2 Fluid Density. Increasing the fluid density generally increased extraction efficiency. The highest efficiency was obtained at 0.90 g mL^{-1}, which was the highest CO_2 density obtainable at 50 °C with our instrument. The trend was similar at two different extraction temperatures, 70 °C (results not shown) and 50 °C (Table 6). Our results are in agreement with the generalization of previous workers that higher fluid densities tend to increase the solvating power of the supercritical fluid (Hawthorne, 1990) and generally improve the extraction of polar compounds (Knipe et al., 1992).

Static and Dynamic Extraction Time. Supercritical fluid extraction can be performed in either a static or a dynamic mode. In the majority of applications, a combination of the two modes is used (Hawthorne, 1990; Nam and King, 1994; Locke, 1993; Lopez-Avila and Dodhiwala, 1993; Reddy and Locke, 1994). When combination mode is used, static extraction is usually a small equilibration/extraction step wherein the CO_2 fluid is recycled through the sample for a period of time to allow the extraction fluid to fully penetrate the sample and dissolve the analyte. The second extraction step, dynamic extraction, involves fresh fluid CO_2 continuously flowing through the sample chamber enabling further analyte dissolution and removal from the sample chamber. For cyanazine recovery, increasing the static extraction time from 3.0 to 10.0 min did not significantly affect the extraction efficiency, although the variability in the results was slightly lower at higher

Table 8. Recovery of Cyanazine Metabolites from an Inert Matrix,[a] Using SFE Conditions[b] Optimized for Cyanazine

recovery (% of applied)		
metabolite[c]	mean[d]	SD
ii	36.7	2.39
iii	<5	—
iv	72.4	2.10
v	72.8	2.35
vi	6.8	0.26

[a] Kimwipe tissue paper. [b] SFE conditions: CO_2 fluid density, 0.90 g mL^{-1}; extraction temperature, 50 °C; 20.0 and 6.0 min dynamic and static extraction times, respectively. [c] 10.0 μg samples of each metabolite, see Materials and Methods. [d] Means of three replications.

equilibration times (Table 7). Increasing the dynamic extraction time from 10.0 to 20.0 min improved recoveries while further increases to 35.0 min were not beneficial (Table 7). With a static extraction time of 6.0 min, the optimum extraction time was 26.0 min.

One of the main advantages of SFE is shorter sample preparation times as compared to conventional extraction procedures. It is of interest to know the minimum time required to obtain satisfactory recovery. Allowing time for initial pressure and temperature attainment, postextraction elution from the solid-phase trap, and depressurization of the system, cyanazine could be extracted from a 3.0 g soil matrix in approximately 45–50 min with high efficiency. This is a considerable savings in time compared to conventional extraction times which could range from a few hours to overnight extractions. The extract is automatically collected in three 1.0 mL aliquots, of which, the first 1.0 mL contains more than 98% of the analyte. An additional advantage of the SFE procedure, particularly when dealing with field samples, is the concentration of the analyte during extraction, compared to conventional extraction procedures which use a much higher solvent/soil ratio and time-consuming evaporation steps.

SFE of Cyanazine Metabolites. Results from a set of triplicate extractions of cyanazine metabolites (**ii**–**vi**) from Kimwipe tissue paper are shown in Table 8. Reasonable recoveries were obtained for moderately polar deethylcyanazine (**v**) and deisopropylatrazine (**iv**). The amide (**ii**) recovery was less than 50%. Efforts made to improve negligible recoveries of the hydroxy acid (**iii**) and the deethyl amide (**vi**) by increasing the percentage of water in the modifier and by acidifying the water were not successful. There was no postextraction loss or degradation of the metabolites since the unextracted materials were almost quantitatively recovered by extraction with 1:1 methanol/water solvent. Detailed study needs to be conducted with respect to the modifier, and possibly the extraction temperature, if the main analyte of interest is other than cyanazine.

Soil Incubation Studies. Incubation of soil at 25 °C for 35 days produced substantial amounts of several metabolites (Table 9). The total cyanazine (cyanazine + metabolites) extractability decreased as the time progressed, from 92% on day 1 to 66% on day 35 (Table 10). It was not possible to identify the unextractible ^{14}C. Although 4:1 methanol/water extraction solvent was as effective as 1:1 methanol/water for conventional extraction of cyanazine (Table 2), the extraction efficiencies for polar metabolites were very poor with the 4:1 methanol/water solvent. The extractabilities of metabolites by SFE were substantially lower than those by the conventional procedure (Table 9). Once again, there was no loss or degradation of the metabolites during the SFE since the unextracted (by SFE) fractions of the metabolites were extracted with 1:1 methanol/water solvent to yield a combined extraction efficiency equivalent to the conventional procedure alone (Tables 9 and 10). Under the present set of conditions optimum for cyanazine, the extractabilties of the metabolites from soil by SFE are parallel to the recoveries from inert matrix (Table 8). More work needs to be done to optimize the SFE conditions for the polar metabolites. However, if the major components in soil are cyanazine or its moderately polar metabolites **iv** or **v**, SFE can be utilized as efficiently as the conventional procedure with savings in time and eliminating the additional concentration step.

Summary and Conclusions. Cyanazine was quantitatively recovered by SFE from an inert matrix (Kimwipe tissue paper) with high reproducibility. Effects of addition of a modifier, sample mass, extraction temperature, CO_2 fluid density, and static and dynamic extraction times on the recovery of cyanazine from treated Dundee silty clay loam soil were investigated. Addition of 20% (v/w) methanol/water (1:1) modifier directly to the sample resulted in a significant improvement in recovery compared to the extraction from dry soil. Although the variation of extraction temperature between 40 and 70 °C had little or no effect on the recovery, higher temperatures affected extraction adversely. Increasing the CO_2 fluid density generally resulted in improved extraction efficiency (highest recovery at a CO_2 density of 0.90 g mL^{-1}). Static extraction times longer than 6.0 min and dynamic extraction times longer than 20.0 min did not significantly improve cyanazine recovery. Although other solid-phase trapping materials were not investigated, the ODS trap was found to be quite efficient. SFE

Table 9. Distribution of Metabolites of Cyanazine[a] in Extracts from Treated Soil, Incubated for 35 Days at 25 °C

	% of individual metabolites[b]				
extraction procedure	**i**	**ii**	**iii**	**v**	**vi**
			Conventional		
HPLC	65.6 (1.41)	2.5 (0.70)	23.6 (0.78)	5.7 (1.10)	2.6 (0.40)
TLC	70.7 (4.21)	1.3 (1.50)	18.1 (3.33)	8.7 (1.74)	1.4 (0.90)
			SFE		
HPLC	69.3 (1.92)	0.7 (0.15)	0.0	5.6 (1.07)	0.03 (0.06)
TLC	68.7 (3.03)	0.0	0.0	8.55 (2.48)	0.0
			Post-SFE Conventional[c]		
HPLC	1.0 (0.25)	2.2 (0.38)	18.6 (1.84)	0.6 (0.20)	2.0 (0.17)
TLC	1.7 (0.26)	2.5 (0.64)	13.8 (1.64)	1.1 (0.63)	3.1 (1.06)

[a] Nonlabeled and ^{14}C-ring-labeled cyanazine-treated soil samples (3.0 g), extracts analyzed by HPLC and TLC. [b] Means of at least six replications. Numbers in the parentheses are standard deviations. For SFE and post-SFE conventional extraction, inividual numbers are percentages of combined total recovery. [c] Spent soil samples from SFE, extracted by conventional method.

Table 10. Recovery of Cyanazine[a] and Its Metabolites from Treated Soil, Incubated for 35 Days at 25 °C

	total recovery (% of applied)[b]			
	^{14}C counting		HPLC	
extraction procedure	mean[d]	SD	mean[d]	SD
conventional	67.9	3.19	66.0	2.31
SFE	49.1	4.22	50.9	3.95
post-SFE conventional[c]	15.7	2.35	16.5	2.47

[a] Nonlabeled and ^{14}C-ring-labeled cyanazine-treated soil samples (3.0 g), extracts analyzed by HPLC and assyed for radioactivity by scintillation counting. [b] Sum of recoveries of all metabolites. [c] Spent soil samples from SFE extracted by conventional method. [d]Means of at least six replications.

recoveries equivalent to the conventional procedure were obtained for cyanazine while the conditions were less efficient for the metabolites. More work needs to be done to optimize the process for the metabolites. However, if the major components in soil are cyanazine or deethylcyanazine, the advantages inherent in the procedure (single and faster extraction step, concentration of the extract, lower risk of personnel exposure, and lower solvent waste generation) make supercritical fluid extraction an attractive alternative to the conventional procedure.

LITERATURE CITED

Benyon, K. I. The analysis of crops and soils for the triazine herbicide cyanazine and some of its degradation products. *Pestic. Sci.* **1972**, *3*, 389−400.

Benyon, K. I.; Stoydin, G.; Wright, A. N. The breakdown of the triazine herbicide cyanazine in soils and maize. *Pestic. Sci.* **1972a**, *3*, 293−305.

Benyon, K. I.; Bosio, P., Elgar, K. E. The analysis of crops and soils for the triazine herbicide cyanazine and some of its degradation products. *Pestic. Sci.* **1972b**, *3*, 401−408.

Blumhorst, M. R.; Weber, J. B. Cyanazine dissipation as influenced by soil properties. *J. Agric. Food Chem*. **1992**, *40*, 894−897.

Blumhorst, M. R.; Weber, J. B. Chemical versus microbial degradation of cyanazine and atrazine in soils. *Pestic. Sci.* **1994,** *42*, 79−84.

Brown, N. P. H.; Furmidge, C. G. L.; Grayson, B. T. Hydrolysis of the triazine herbicide, cyanazine. *Pestic. Sci.* **1972,** *3*, 669−678.

Calvey, E. M.; Matsuik, J. E.; White, K. D.; Betz, J. M.; Block, E.; Littlejohn, M. H.; Naganathan, S.; Putman, D. Offline supercritical fluid extraction of thiosulfinates from garlic and onion. *J. Agric. Food Chem*. **1994,** *42*, 1335−1341.

Corcia, A. D. and Marchetti, M. Method Development for monitoring pesticides in environmental waste waters: Liquid−solid extraction followed by liquid chromatography. *Environ. Sci. Technol.* **1992**, *26*, 66−74.

Cotterill, E. G. The efficiency of methanol for extraction of some herbicide residues from soil. *Pestic. Sci.* **1980,** *11*, 23−28.

Hawthorne, S. B. Analytical scale supercritical fluid extraction. *Anal. Chem*. **1990,** *62*, 633−642A.

Hawthorne, S. B.; Riekkola, M. L.; Serenius, K.; Holm, Y.; Hiltunen, R.; Hartonen, K. Comparison of hydrodistillation and supercritical fluid extraction for the determination of essential oils in aromatic plants. *J. Chromatogr.* **1993,** *634*, 297−308.

Howard, A. L.; Taylor, L. T. Quantitative supercritical fluid extraction of sulfonylurea herbicides from aqueous matrices via solid-phase extraction disks. *J. Chromatogr. Sci.* **1992,** *30*, 374−382.

Huston, C. K.; Ji, H. Optimization of the analytical supercritical fluid extraction of cloves via an on-column interface to an ion trap GC/MS system. *J. Agric. Food Chem*. **1991**, *39*, 1229−1233.

Janda, V.; Steenbeke, G.; Sandra, P. Supercritical fluid extraction of s-triazine herbicides from sediment. *J. Chromatogr.* **1989**, *479*, 200−205.

King, J. W. Fundamentals and applications of supercritical fluid extraction in chromatographic science. *J. Chromatogr. Sci.* **1989**, *27*, 355−364.

King, J. W.; Hopper, M. L.; Luchtefeld, R. G.; Taylor, S. L.; Orton, W. L. Optimization of experimental conditions for the supercritical fluid extraction of pesticide residues from grains. *J. AOAC Int.* **1993,** *76*, 857-864.

Knipe, C. R.; Gere, D. R.; McNally, M. E. Supercritical fluid extraction: Developing a turnkey method. *In Supercritical Fluid Technology*; Bright, F., McNally, M. E., Eds; ACS Symposium Series 488; American Chemical Society; Washington, DC, 1992; pp 251−265.

Lee, H. B.; Peart, T. E.; Hong-You, R. L. *In situ* extraction and derivatization of pentachlorophenol and related compounds from soils using supercritical fluid extraction system. *J. Chromatogr.* **1992,** *605*, 109−113.

Locke, M. A. Supercritical CO_2 fluid extraction of fluometuron herbicide from soil. *J. Agric. Food Chem*. **1993,** *41*, 1081−1084.

Locke, M. A.; Pothuluri, J. V.; Moorman, T. B.; Harper, S. S. Efficiency of methanol: water solutions for metribuzin extraction from selected soils. *Soil Sci. Plant Anal.* **1990**, *21*, 2141−2152.

Lopez-Avila, V.; Dodhiwala, N. S. Developments in the supercritical fluid extraction of chlorophenoxy acid herbicides from soil samples. *J. Agric. Food Chem*. **1993**, *41*, 2038−2044.

McNally, M. E.; Wheeler, J. R. Supercritical fluid extraction coupled with supercritical fluid chromatography for a separation of sulfonylurea herbicides and their metabolites from complex matrices. *J. Chromatogr.* **1988a,** *435*, 63−71.

McNally, M. E.; Wheeler, J. R. Increasing extraction efficiency in supercritical fluid extraction from complex matrices: Predicting extraction efficiency of diuron and linuron in supercritical fluid extraction using supercritical fluid chromatographic retention. *J. Chromatogr.* **1988b**, *447*, 53−63.

Muir, D. C. G.; Baker, B. E. The disappearance and movement of three triazine herbicides and several of their degradation products in soil under field conditions. *Weed Res.* **1978**, *18*, 111−120.

Murugaverl, B.; Gharaibeh, A.; Voorhees, K. J. Mixed sorbent cleanup during supercritical fluid extraction of three carbamate pesticides in tissues. *J. Chromatogr. A* **1993**, *657*, 223−226.

Nam, K. S.; King, J. W. Supercritical fluid extraction and enzyme immunoassay for pesticide detection in meat products. *J. Agric. Food Chem*. **1994,** *42*, 1469−1474.

Reddy, K. N.; Locke, M. L. Supercritical fluid extraction of imazaquin from soil. W*eed Sci.* **1994**, *42*, 249−253.

Rochette, E. A.; Koskinen, W. C. Supercritical carbon dioxide for determining atrazine sorption by field-moist samples. *Soil Sci. Soc. Am. J.* **1996**, *60*, 453−460.

Sharma, A. K.; Procopczyk, B.; Hoffmann, D. Supercritical fluid extraction of moist snuff. *J. Agric. Food Chem*. **1991**, *39*, 508−510.

Sirons, G. J.; Frank, R.; Sawyer, T. Residues of atrazine, cyanazine, and their phytotoxic metabolites in a clay loam soil. *J. Agric. Food Chem*. **1973**, *21*, 1016−1020.

Snyder, J. M.; King, J. W.; Rowe, L. D.; Woerner, J. A. Supercritical fluid extraction of poultry tissues containing incurred pesticide residues. *J. AOAC Int.* **1993**, *76*, 888−892.

Sugiyama, K.; Saito, M. Simple microscale supercritical fluid extraction system and its application to gas chromatography−mass spectrometry of lemon peel oil. *J. Chromatogr.* **1988,** *442*, 121−131.

Thurman, E. M.; Meyer, M.; Pomes, M.; Perry, C. A.; Schwab, A. P. Enzyme-linked immunosorbent assay comppared with gas chromatography/ mass spectrometry for the determination of triazine herbicides in water. *Anal. Chem*. **1990**, *62*, 2043−2048.

van-der Velde, E. G.; de Haan, W.; Liem, A. K. D. Supercritical fluid extraction of polychlorinated biphenyls and pesticides

from soil. Comparison with other extraction methods. *J. Chromatogr.* **1992,** *626,* 135−143.
van-der Velde, E. G.; Dievorst, M.; Swart, C. P.; Ramlal, M. R. Optimization of supercritical fluid extraction of organochlorine pesticides from real soil samples. *J. Chromatogr. A* **1993,** *683,* 167−174.

Received for review June 6, 1996. Revised manuscript received December 12, 1996. Accepted December 19, 1996.[⊗] We sincerely thank Dr. Susan Hausman of DuPont Agricultural Products, Wilmington, DE, for providing the ^{14}C-radiolabeled cyanazine and the metabolite standards for this work.

JF960397J

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.