Ethanol-Modified Subcritical Water Extraction Combined with Solid-Phase Microextraction for Determining Atrazine in Beef Kidney

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The determination of the levels of pesticides in food products has prompted the development of sensitive and rapid methods of analysis that are solvent-free or utilize solvents that are benign to the environment and laboratory worker. In this study we have developed a novel extraction method that utilizes ethanol-modified subcritical water in combination with solid-phase microextraction (SPME) for the removal of atrazine from beef kidney. In situ sample cleanup was achieved using the technique of matrix solid-phase dispersion. A cross-linked polymer, XAD-7 HP, was utilized as a dispersing material for kidney samples. Subcritical water extractions were performed with a pressurized solvent extraction unit at 100 °C and 50 atm. Experimental parameters investigated were the volume of solvent and amount of modifier required for the complete extraction of atrazine and optimization of the extraction time. It was determined that 30% ethanol in water (v/v) is adequate for the complete extraction of atrazine. A Carbowax-divinylbenzene SPME fiber was used to sample the aqueous extracts. Analysis of the fiber contents was by ion-trap GC/MS utilizing the single ion mode. The total time of analysis for a single kidney sample is 90 min. The average percent recoveries from samples spiked to the concentrations of 2 and 0.2 μg/g were 104 and 111, respectively. The average relative standard deviations were 10 and 9, respectively. The method limit of detection for beef kidney spiked with atrazine was found to be 20 ng/g of sample.

Keywords: *Subcritical water; pressurized extraction; ASE; matrix solid-phase dispersion (MSPD); solid-phase microextraction (SPME); triazines; atrazine*

INTRODUCTION

Concern for food quality has emphasized the continuous need for sensitive and rapid methods of analysis for food contaminants that are also benign to the environment and laboratory worker. The presence of pesticides in food products continues to be of increasing concern to the general public, resulting in the introduction of legislation such as the Food Quality Protection Act of 1996 (FQPA), which requires reassessment of the levels of pesticide residues allowed in foods. Of specific interest is the class of weed herbicides known as triazine pesticides, of which 40−55 million kilograms are used each year in the United States in agricultural production (*1*). Atrazine is the most widely used of all the triazines. In 1992, atrazine was applied to two-thirds of all cultivated crops in the United States (*2*). This widespread and persistent usage raises concern since atrazine is considered as a possible (group C) carcinogen by the U.S. Environmental Protection Agency (*3*).

In response to the need for newer extraction methods, several alternative analytical methods have been developed to reduce the amount of toxic, organic solvents that are used in conventional extraction procedures such as liquid−liquid, Soxhlet, solid-phase, or liquid−solid extraction methods. For example, supercritical carbon dioxide (SC CO_2) has been used as an alternative, environmentally benign extraction agent for the removal of contaminants from food (*4*−*6*). Although the method of supercritical fluid extraction (SFE) using SC CO_2 addresses the issues of environmental and health safety, it is not the only option for the direct analysis of contaminants in meat samples. In addition, the nonpolar nature of SC CO_2 can limit its selectivity during the analytical extraction of meat samples, resulting in the co-extraction of lipids which necessitates using additional steps for sample cleanup.

An alternative, low-solvent extraction method that has been used in the food industry is the solid-phase microextraction technique (SPME), based on the partitioning of analytes to a polymeric bonded phase coated on a fused silica fiber. For sampling purposes, the fiber is either directly immersed in a liquid (aqueous) sample or placed in the headspace over the sample for collecting sufficiently volatile analytes. The fiber with the collected sample is then placed in the hot injector of a gas chromatograph and thermally desorbed. SPME as a one-step sample preparation technique for water samples has also been used for the analysis of aqueous food samples (*7*−*10*).

SPME of solid food samples is more difficult, but can still be achieved by several means. The sample can be mixed directly with water to form an aqueous suspension or it can be first extracted with a suitable organic solvent via a conventional liquid−solid extraction method. The organic extract can then be diluted with water for SPME analysis. The first approach is more difficult as it requires a matrix that can be easily dispersed in

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water, for example, in the analysis of soil contaminants (*11*). The latter approach is more practical, but has the disadvantage that organic solvents are reintroduced to the analytical procedure.

Total elimination of toxic solvents from the SPME preextraction step for solid samples can be achieved by using subcritical or "hot" water as the extraction agent. The advantages of using subcritical water are its nontoxic nature, low cost, and the fact that it can be readily obtained and disposed of. Although the dielectric constant (ϵ) of ambient water is very high ($\epsilon = 78.5$), its polarity is lowered by increasing the temperature to values between 50 and 250 °C. In this way, the dielectric constant of water can be decreased to a value as low as 27 at 250 °C (*12*).

The combination of subcritical water extraction (SbWE) and SPME has been utilized previously for the removal of pesticides and other contaminants from environmental solids (*7*, *13*−*15*). SbWE extracts have also been trapped on solid-phase sorbents or have been analyzed on-line by HPLC (*16*−*20*). It should be noted that subcritical water extraction has also been used in the food industry for the extraction of flavor and fragrance compounds from plant material (*21*, *22*), fungicides from vegetable food samples (*23*), and mycotoxins from corn products (*24*). However, we are not aware of any studies that have utilized subcritical water for the extraction of meat products.

Solubility experiments performed in our laboratory have indicated that subcritical water extraction of triazine pesticides is feasible at lower temperatures if cosolvents are used in conjunction with an adjustment of the extraction temperature in reducing the polarity of water (*25*). This approach is preferred for solutes that are thermally labile. Ethanol is a suitable cosolvent for the extraction of triazine pesticides as it is nontoxic and can be safely disposed of along with the rest of the aqueous media. Lawrence et al., for example, have demonstrated the effectiveness of ethanol as a cosolvent during the subcritical water extraction of fumonisins from contaminated corn products (*24*). In addition, ethanol has also been used as cosolvent for the removal of surfactants from sludge samples (*26*).

The inherent difficulty in the extraction of meat samples is the co-extraction of matrix components that can include fatty acids, glycerides, sterols, and protein matter. The resultant extracts are usually highly colored and can be quite turbid. Sample cleanup can be achieved in situ with the technique of matrix solid-phase dispersion (MSPD), which has been successfully applied to the removal of drugs and pesticides from biological matrices such as animal tissues and dairy products (*27*−*30*). During an MSPD procedure, the sample matrix is combined with a polymer resin that is bound to a solid support, such as C_{18} reversed-phase material. Grinding the sample in the presence of solid C_{18}-modified silica facilitates disruption of the sample matrix. Complete disruption is achieved by dissolution and dispersion of the sample into the bound resin. The final result is that the entire dispersed sample becomes a unique chromatographic phase from which either the analytes or matrix components can be selectively eluted. Although we are not aware of any reports that have employed MSPD for sample cleanup during a subcritical water extraction procedure, subcritical water has been investigated as the mobile phase in the elution of organic solutes from different polarity sorbents (*31*−*33*).

In the present work, we have utilized the cross-linked polymer, XAD-7 HP, for the MSPD cleanup of kidney samples. This appears to be the first study utilizing an acrylic polymer as the dispersant in an MSPD procedure. By integrating the MSPD procedure, aqueous extracts from the SbWE of kidney samples can be analyzed directly by SPME using a Carbowax-divinylbenzene (CW-DVB) fiber. The CW-DVB fiber has previously been shown to be effective in the removal of atrazine from aqueous solution (*7*, *8*).

MATERIALS AND METHODS

Materials and Instrumentation. Subcritical water extractions of kidney samples were performed with an ASE 200 accelerated solvent extractor (Dionex Corp.; Sunnyvale, CA). An ASE 200 solvent controller was used to deliver both pure and ethanol-modified water. An 11 mL ASE extraction cell was used for all experiments. Aqueous extracts from the ASE were sampled with a 65 μm Carbowax-divinylbenzene (CW-DVB) solid-phase microextraction (SPME) fiber (Supelco; Bellefonte, PA) using 2 mL sampling vials (Supelco; Bellefonte, PA). The fibers were desorbed in the injector of a Varian 3600 gas chromatograph (GC) that was connected in tandem with a Varian Saturn 4D ion trap mass spectrometer (MS) (Varian, Inc.; Palo Alto, CA). The GC was equipped with a DB-5 column (30 m × 0.25 mm i.d.; 0.10 μm) (J&W Scientific, Inc.; Folsom, CA), and the injector contained a splitless glass inlet liner for SPME (0.75 mm i.d.) (Supelco; Bellefonte, PA). Qualitative analysis of the lipid content of swine kidney extracts was performed with a Dionex Scientific Series 600 supercritical fluid chromatograph (SFC) (Dionex Corp.; Sunnyvale, CA) using SC CO_2 as the mobile phase. The SFC was equipped a Dionex SB-Phenyl-50 column (10 m × 0.10 mm i.d.; 0.5 μm film thickness) and a flame ionization detector.

The analyte atrazine (Chem Service, Inc.; West Chester, PA) had a purity of 99.0%. The ethanol (Aaper Alcohol and Chemical Co.; Shelbyville, KY) was absolute 200 proof. Distilled water was passed through a MilliQ water system for deionization prior to use. Amberlite XAD-7 HP resin (Supelco; Bellefonte, PA) was used to disperse the kidney samples, and the diatomaceous earth (Hydromatrix) that was used in the experiments was from Varian Corp. (Palo Alto, CA). Methanol (HPLC grade) (Fisher Scientific; Pittsburgh, PA) was used to wet the XAD-7 HP polymer. Beef and swine kidney were obtained from a local retail outlet. Whole kidneys were homogenized in a blender (Waring Products; New Hartford, CT) prior to being frozen.

Matrix Solid-Phase Dispersion. The XAD-7 HP resin was wetted prior to dispersing the kidney samples since the polymer can become dry during shipping and storage. To wet the polymer, an appropriate amount of dry XAD-7 HP was weighed into a clean beaker and the resin covered with methanol for 15 min. The methanol was then decanted, and the resin was further soaked in water for 5 min. The resin was then rinsed three times with water to ensure all the methanol had been removed.

To prepare a beef kidney sample, 1 g of Hydromatrix was weighed into a plastic weighing dish. The beef kidney was slightly thawed so that a 0.5 g portion could be sampled on top of the Hydromatrix. The kidney was then spiked by flooding the surface of the meat with a 1 μL aliquot of atrazine prepared in methanol. The kidney samples were spiked to a concentration of 2 μg/g kidney unless otherwise specified. The samples were allowed to equilibrate for 30 min to allow sufficient time for the solvent to evaporate and to permit the standard to impregnate the meat sample. The sample was not allowed to become completely dry, as this made the kidney very difficult to disperse. Following the above equilibration time, an additional 1 g of Hydromatrix was placed on top of the meat. The contents of the weighing dish were then placed into a mortar, to which was added the wetted XAD-7 HP resin. The entire sample was dispersed with the pestle until the sample was powder-like and there were no visual traces of

kidney tissue. Swine kidney samples that were assessed for qualitative purposes were prepared in the same manner, both with and without the addition of XAD-7 HP.

Pressurized Solvent Extraction. A cellulose fiber was pressed into an 11 mL ASE extraction cell before the cell was filled with the dispersed kidney sample. An additional amount of diatomaceous earth was placed in the mortar, which was swept with the pestle to remove any trace amounts of the kidney sample. This was also added to the extraction cell, and any remaining void volume in the cell was filled with unground Hydromatrix.

Unless otherwise specified, the SbWE extractions were performed at 100 °C and 50 atm utilizing a 0 min preheat step, a 5 min heating period, three 10 min static extraction cycles, a 50% flush volume (the flush volume is defined as a percentage of the total cell volume), and a 1 min purge time. The amount of ethanol that was delivered by the solvent controller to modify the water varied between 0 and 30%.

Sample Analysis. The beef kidney extracts from the ASE were sampled directly by SPME by placing a 1.5 mL aliquot in a sampling vial. The SPME sampling parameters were taken from the reference by Hernandez et al. for the determination of atrazine in water and aqueous soil extracts (*7*). This involved using a 65 μm CW-DVB fiber to sample the extracts over a 30 min absorption time. The fiber was placed off-center in the sampling vial so that the sample flowed perpendicular to the fiber axis. The SPME fiber was then desorbed for 5 min in the GC injector, heated to 250 °C.

A GC/MS chromatographic method based on full-scan detection was initially applied to injection of standards to determine the retention time and characteristic fragmentation ions of atrazine. All other analyses were performed in the single ion mode (SIM) by scanning the principle ion at 215 amu. SPME standards for atrazine were prepared by spiking blank kidney extracts with 0.5−2 μL of a stock solution prepared in methanol or water at the appropriate concentration. In this fashion we were able to account for the sorption of matrix components by the SPME fiber during the analysis of atrazine from beef kidney samples.

Initial qualitative analyses of blank swine kidney extracts using pure water were performed to determine their lipid and protein content. For lipid determination, the extracts were subjected to a conventional liquid−liquid extraction procedure using 2:1 ether/methanol as the extraction solvent. The organic extract was then analyzed by supercritical fluid chromatography. The pressure method that was utilized for analysis was as follows: Initial pressure 100 atm; hold 5 min; increase pressure 5 atm/min to 150 atm; increase pressure 2 atm/min to 180 atm; increase pressure 5 atm/min to 320 atm. The column was held at 100 °C. Standards containing varying amounts of palmitic, stearic, and oleic fatty acids in addition to cholesterol and C18:0 mono-, di-, and triglycerides were used to speciate the contents of the extract. The Kjeldahl method was applied to the aqueous kidney extracts for the determination of protein content.

RESULTS AND DISCUSSION

XAD-7 HP for Matrix Solid-Phase Dispersion. Preliminary experiments were performed on swine kidney to investigate the retention of matrix components when using the XAD-7 HP resin as a dispersant in the MSPD procedure. To determine if any major matrix components were extracted with subcritical water, kidney samples that were dispersed with only diatomaceous earth were first extracted with the ASE unit using pure water as the solvent at 100 °C and 50 atm. It was determined by supercritical fluid chromatography analysis that free fatty acids, cholesterol, and triglycerides were removed from swine kidney with subcritical water. In addition, the Kjeldahl method confirmed the presence of small amounts of protein in the extracts. The resultant extracts were also assessed visually. The solutions were turbid and highly colored, which indicated that they contained appreciable quantities of matrix components.

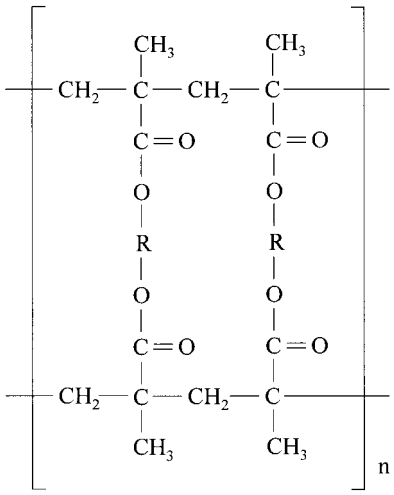

Figure 1. Structure of Amberlite XAD-7 HP acrylic polymer.

These amounts of lipid and protein material in the aqueous extracts were significantly reduced when the polymer XAD-7 HP was used to disperse kidney samples. The XAD-7 HP resin is a methacrylate polymer (illustrated in Figure 1) that has intermediate polarity with the potential of participating in both hydrophobic and hydrophilic interactions with the solutes, matrix components, and the solvent. The hydrophilic properties of XAD-7 HP also made the polymer water-wettable during the subcritical water extraction procedure. It was determined that the amount of diatomaceous earth and XAD-7 HP required for cleanup of both swine and beef kidney samples was four times the mass of the kidney sample. Using these extraction conditions, the aqueous extracts were not turbid, only pale yellow in color, and devoid of any emulsion formations. It must be emphasized that this result was obtained only when the XAD-7 HP was rinsed completely with water following the methanol-wetting step, otherwise turbid extracts were the result.

It has been shown that solid silica aids in disrupting the sample matrix during an MSPD procedure, as it provides a surface for shearing the matrix during sample dispersion (*29*). However, it would appear that adequate disruption of the sample matrix can also be achieved with XAD-7 HP. The mechanical process of grinding the kidney sample in the presence of XAD-7 HP and diatomaceous earth using a mortar and pestle was sufficient to permit retention of the matrix components in this work.

Ethanol-Modified Water. To determine the solvent strength that is required for the removal of atrazine from beef kidney dispersed with XAD-7 HP, MSPD-aided extractions were first performed with pure subcritical water as the eluent at 100 °C. It was immediately apparent that a stronger solvent was required to elute the pesticide from the dispersed matrix, as there was no recovery of the analyte with pure water at this temperature. Ethanol-modified water was therefore examined as an alternative media, since it was previously determined that the solubility of atrazine in subcritical water is increased by an order of magnitude when 20 wt % ethanol is added at 100 °C (*25*). Hence, the effect of the amount of ethanol in subcritical water on atrazine recovery from kidney was determined at 100

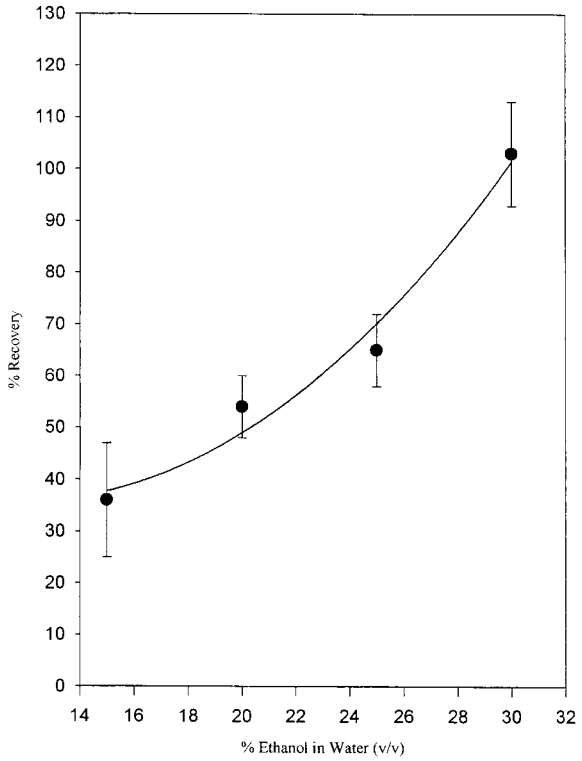

Figure 2. Recovery of atrazine from beef kidney dispersed with XAD-7 HP as a function of the amount of ethanol in subcritical water at 100 °C and 50 atm.

°C. As shown in Figure 2, complete recovery of atrazine is obtained with 30% ethanol in water (v/v). Each data point in Figure 2 represents the average from the extraction of three different kidney samples. Duplicate analysis of each of these sample extracts was performed by GC/MS.

The ratio of 30% ethanol in water (v/v) corresponds to a solvent mixture composed of 25 wt % ethanol, which has a dielectric constant of 43 at 100 °C (extrapolated from the data in ref 12). The presence of ethanol in the water therefore causes the modified solvent to be significantly less polar than pure water at the same temperature ($\epsilon = 55$). In addition, ethanol interacts with both the solute (*25*) and the XAD-7 HP sorbent during an extraction. The polymer has been shown to readily take up organic solvents such as ethanol (*34*). These favorable interactions enable atrazine to be displaced from the polymer and facilitate elution of the analyte from the dispersed sample. It was evident that higher amounts of matrix components were also removed from the dispersed sample when ethanol was used as modifier. Nonetheless, the analyte could be effectively concentrated on the Carbowax-divinylbenzene SPME fiber. GC/MS analysis of the desorbed fibers showed good chromatographic resolution of atrazine from the coextracted matrix components, as is illustrated in the chromatogram in Figure 3.

Good fiber stability was also observed during SPME analysis of the ethanol-modified water extracts. We were able to use a single fiber for more than 60 analyses. However, it was apparent that ethanol was also taken up by the fiber. This resulted in reduced partitioning of atrazine to the CW-DVB fiber, as evidenced by a corresponding decrease in the peak area for atrazine's single ion at 215 amu as the amount of ethanol in the extracts increased. To demonstrate the effect of ethanol on the sorption of atrazine, several standards with the

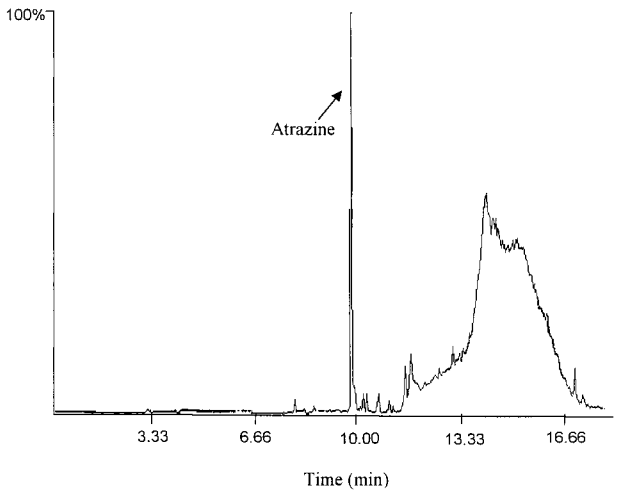

Figure 3. GC/MS chromatogram for atrazine from an aqueous extract of beef kidney in the single-ion mode (215 amu).

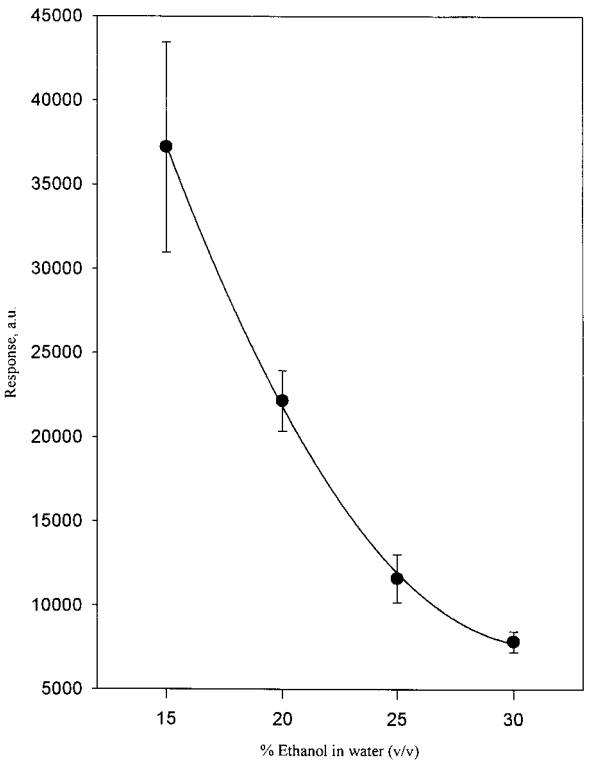

Figure 4. Effect of ethanol concentration on the peak area of atrazine from an aqueous kidney extract following sampling with a CW-DVB SPME fiber ($n = 3$).

concentration of 0.074 μg/mL were prepared using blank tissue extracts. The amount of ethanol in the kidney extracts varied from 15 to 30% (v/v). Figure 4 shows that the GC/MS response for the desorbed CW-DVB fiber is declining as the ethanol content in the kidney extracts is increasing. The detected signal is plotted in arbitrary units (ionic current). This observation is consistent with those of Urruty and Montury, who had previously determined that an ethanol concentration of up to 15% reduces the partitioning of pesticides into a PDMS fiber (*9*). However, despite the take-up of ethanol by the CW-DVB fiber, a satisfactory limit of detection of 1 ng/mL of atrazine in the aqueous extracts was achieved using 30% ethanol in water (v/v) as the extraction solvent.

Flush Volume. The total volume of solvent that is used to extract a sample during the ASE extraction

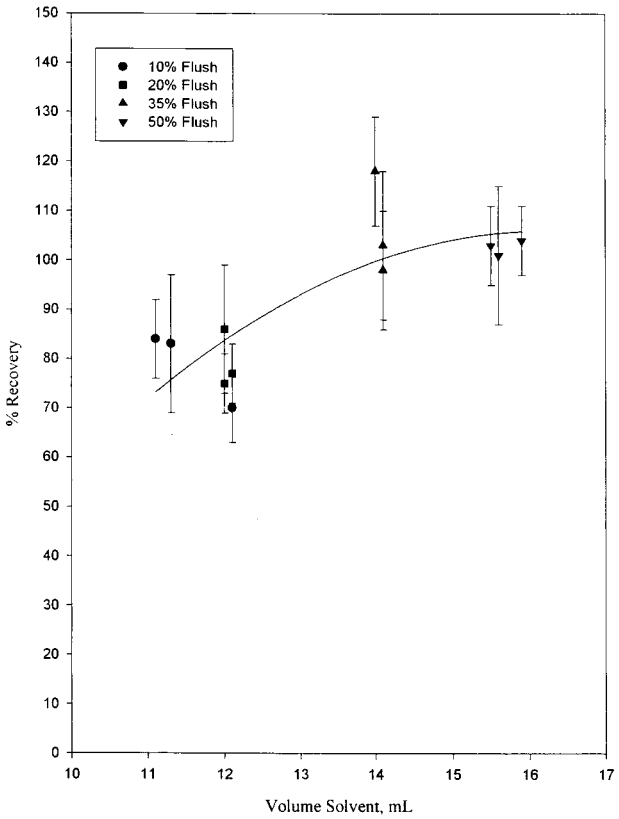

Figure 5. Recovery of atrazine from beef kidney as a function of the volume of extraction solvent and different flush volumes.

procedure is determined by the volume of the ASE extraction cell, the size of the sample, and the amount of solvent used to elute or "flush" the analyte from the cell following the static extraction period. During an ASE procedure, it is common practice to ensure that the extraction cell is completely filled with the sample or that the void volume is taken up with an inert material, to minimize solvent consumption. In this procedure, the control of the flush volume provided a means to ensure complete elution of atrazine from beef kidney dispersed with XAD-7 HP, while at the same time minimizing excess dilution of the extract. This proved important during subsequent SPME analysis as greater sensitivity was obtained for extracts in which the solute's concentration was maximized.

To determine the optimum flush volume, the percent flush volume was varied from 10 to 50%. Three samples were extracted at each extraction condition. Each extract was analyzed in triplicate by GC/MS. Because the total volumes of the extracts were not identical under the same extraction conditions, the recovery data for each individual sample were plotted separately as a function of total volume as shown in Figure 5. Figure 5 indicates that quantitative recovery of atrazine was obtained for flush volumes as small as 10%, or 1.5 mL. However, a flush volume of 50% is recommended for complete recovery and good reproducibility. This represents a total solvent volume of 15−16 mL. It would appear that atrazine recovery is somewhat variable at a flush volume of 35%. Under this extraction condition, the solvent volume was not always sufficient for complete elution of the analyte.

Extraction Time. To increase sample throughput, the static extraction time was examined for the subcritical water extraction of atrazine from beef kidney.

Table 1: Extraction Parameters for the Removal of Atrazine from Beef Kidney

extraction parameter	value or condition
sample	
beef kidney	0.5 g
diatomaceous earth	2 g
Amberlite XAD-7 HP	2 g[a]
SbWE/ASE	
preheat	0 min
heat	5 min
static	5 min
purge	60 s
no. of cycles	3
T	100 °C
pressure	50 atm
solvent	30% ethanol in water (v/v)
flush volume	50%
SPME	
fiber[b]	65 μm Carbowax-divinylbenzene
absorption time[b]	30 min
desorption time[b]	5 min
desorption temperature	250 °C

[a] Dry weight prior to wetting. [b] From ref 7.

Table 2: Percent Recovery for Atrazine in Beef Kidney

atrazine in beef kidney (μg/g)	% recovery[a] (% R.S.D.)		
	sample 1	sample 2	sample 3
2	104 (14)	103 (8)	104 (7)
0.2	115 (19)	127 (3)	90 (3)

[a] $n = 3$.

Kidney samples were extracted using four different static extraction times with the ASE unit, from 2 to 10 min for each of three extraction cycles. The total extraction times therefore varied from 12 to 36 min, which included a 5 min preheat step and a 1 min sample purge time. There was incomplete recovery of atrazine for just a 2 min static extraction time. Contact times of 3 min or longer are required to ensure complete diffusion and desorption of the analyte from the dispersed matrix. A 5 min static extraction time, or a total extraction time of 21 min, was chosen as a reasonable compromise.

The optimized and referenced parameters for the SbWE of atrazine from beef kidney have been summarized in Table 1. The total time of analysis is approximately 90 min. This total time includes the times required for sample preparation, ASE extraction, SPME concentration, and analysis of the desorbed fiber by GC/MS. The limit of detection for the method was found to be 10 ng atrazine in 0.5 g beef kidney, or 20 ng/g of kidney, which is equal to the tolerance level for atrazine in livestock meat, fat, and meat byproducts (Federal Regulation 40CFR 180.220). This limit of detection is the equivalent to three times the baseline noise when a CW-DVB SPME fiber is used to sample a beef kidney extract.

Table 2 summarizes the recovery data for kidney samples spiked with two different concentrations of atrazine. The data in this table demonstrate that sensitive and reproducible analysis of atrazine in beef kidney is possible using the described extraction method, which combines the techniques of matrix solid-phase dispersion, SbWE using a commercially available ASE system, and solid-phase microextraction.

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