DETERMINATION OF THE ENVIRONMENTAL FATE AND TISSUE DISTRIBUTION OF TRICHLOROETHYLENE AND ITS METABOLITES

by

AMY DIXON DELINSKY

(Under the Direction of Michael G. Bartlett and James V. Bruckner)

ABSTRACT

Trichloroethylene (TCE) is a volatile organic compound commonly found in drinking water supplies as a result of its widespread use as a metal degreaser and dry cleaning solvent. Dichloroacetic acid (DCA) is found in drinking water as a by-product of the chlorination disinfection process and is also a metabolite of TCE formed via the cytochrome P450 oxidative pathway. Exposure to TCE and DCA is of concern, because the two compounds have been shown to cause cancer in laboratory animals. However, doses of TCE and DCA typically given in animal studies are much higher than the levels to which individuals are exposed to environmentally. Efforts to determine the potential carcinogenicity of the two compounds and the extent of DCA formation from TCE *in vivo* have been hindered by difficulty with developing reliable analytical methods. Uncertainty in results obtained from methods using derivitizing reagents containing sulfuric acid have arisen, because sulfuric acid has been shown to convert up to 80% of TCA (another metabolite of TCA) to DCA. This uncertainty has led to doubts as to whether DCA is formed from TCE *in vivo*. Chapter 1, the introduction, describes the layout of the dissertation and reviews methods currently in the literature for the analysis of TCE and DCA and two additional metabolites of TCE, trichloroacetic acid (TCA) and chloral hydrate (CH). Also included in this chapter are means by which to improve analytical methods for TCE and its metabolites. An optimized method for the determination of TCE by SPME-GC/MS is presented

in Chapter 2. Methods for the analysis of DCA by HILIC-LC/MS/MS are presented for drinking water (Chapter 3) and rat blood and tissues (Chapter 4). All of the methods reported in Chapters 2-4 do not require derivitization of the analytes, and should thus mimimize uncertainty in results due to conversion of TCA to DCA. Chapter 4 presents a method for quantitating DCA in biological samples that is proven to not convert TCA to DCA and demonstrates that DCA is formed *in vivo* as a metabolite of TCE.

INDEX WORDS: Trichloroethylene, TCE, Dichloroacetic Acid, DCA, Gas Chromatography, Liquid Chromatography, Mass Spectrometry, Solid Phase Microextraction, Hydrophilic Interaction Liquid Chromatography, GC/MS, LC/MS, SPME, HILIC, Volatile Organic Compounds, VOC, Haloacetic Acids, HAA

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INTRODUCTION AND LITERATURE REVIEW

Trichloroethylene (TCE) is a small lipophilic compound that is commonly found in indoor air and in drinking water. TCE was extensively used as a dry cleaning agent from the 1930s to 1950s and continues to be widely used as a metal degreaser. Due to its widespread use, partial water solubility, and volatility, TCE has been found extensively in the environment. More specifically, TCE has been found in marine sediments, at 42% of the USEPA's Superfund sites, and in 9 to 34 % of US drinking water supplies (Wu and Schaum 2000, Fay and Mumtaz 1996). TCE is of concern, because animal studies have shown that high, chronic doses of the compound cause liver cancer in mice and kidney cancer in rats (ATSDR Toxicological Profile 1997, Bull *et al.* 2000). The current regulatory limit for TCE in drinking water in the United States is 5 ng/mL. While most areas in the United States have levels of TCE below 5 ng/mL, some areas in California and other states have observed levels of TCE as high as 440 ng/mL in well water (Wu and Schaum 2000). The highest exposure to TCE occurs occupationally. However, it is estimated that approximately 10 -13% of the general population has TCE in their blood (Antoine *et al*. 1986, Ashley *et al*. 1994). Individuals are exposed to TCE in drinking water by 1) ingestion, 2) absorption through the skin during bathing and 3) inhalation while using hot water (Wu and Schaum 2000, Weisel *et al*. 1996). Sufficiently high doses of TCE can exert toxic effects such as central nervous system depression, dizziness, headache, cardiac arrhythmia, and possibly cancer, depending on the type, severity, and length of exposure (ATSDR Toxicological Profile 1997, Bull *et al*. 2000).

Dichloroacetic acid, trichloroacetic acid, and chloral hydrate are three metabolites of TCE and certain other halocarbons that are formed via the cytochrome P450 pathway (Lash *et al*. 2000, Merdink *et al*. 1998). DCA and TCA are, in addition, frequently found in drinking water as chlorination disinfection byproducts. Infants and children are administered chloral hydrate as a sedative for dental, minor surgical, and diagnostic procedures. Exposure to DCA, TCA, and CH is of concern because animal studies indicate that these three metabolites, not TCE, are the proximate liver carcinogens in mice (Bull *et al*. 2000, Bull *et al*. 2002, DeAngelo *et al*. 1996, DeAngelo *et al*. 1999). DCA is the only of the above metabolites that has been found to cause cancer in two species, mice and rats (Bull *et al*. 2000, DeAngelo *et al*. 1996, DeAngelo *et al*. 1997). Individuals are exposed to DCA and TCA, both directly via the ingestion of chlorinated drinking water and indirectly by the metabolism of TCE and related solvents. The MCL for five HAAs (DCA, TCA, MCA, MBA, and DBA) as set by the USEPA is 60 ng/mL. DCA is the only one of the 5 HAAs regulated by the USEPA to have a MCLG of zero and a classification of probable human carcinogen (USEPA 1998).

Determining of the human relevance of animal carcinogenicity data and applying them to risk assessment of TCE and its metabolites has been the source of controversy since the mid 1980s. The USEPA is currently reviewing toxicity and toxicokinetic data on TCE and its metabolites in order to revise its cancer and noncaner risk assessments of TCE. Many pharmacokinetic and mechanistic studies are ongoing. In order to obtain useful information about the roles TCE, DCA, TCA, and CH may have in carcinogenesis, it is necessary to have reliable analytical methods. Uncertainty exists with several methods that use sulfuric acid to derivitize TCA and DCA to more volatile forms for GC analysis, because sulfuric acid has been shown to convert up to 80% of TCA in samples to DCA. Several techniques for the analysis of

TCE, DCA, TCA, and to a lesser extent CH are discussed. Different aspects of analytical methods that are addressed include separation techniques, detectors, extraction techniques, method validation, and future directions in the analysis of TCE and its metabolites. An optimized SPME-GC/MS method for the analysis of TCE in blood and tissue samples is presented in Chapter 2. Validated HILIC-LC/MS methods for analysis of DCA are presented for drinking water samples in Chapter 3 and for blood and tissue samples in Chapter 4. Each of the methods presented do not require derivitization with sulfuric acid, minimizing the uncertainty in results due to conversion of TCA to DCA in samples.

SEPARATION TECHNIQUES

Overview. Separation techniques are typically combined with some type of detection for the analysis of TCE, DCA, TCA, and CH. This section discusses the different types of separation procedures used in the analysis of TCE and its metabolites. Gas chromatography (GC) is by far the most commonly used separation for the analysis of TCE. GC and HPLC are both used frequently in the analysis of DCA and TCA. The main types of chromatography used for HPLC analysis will be discussed and are as follows: ion-pair chromatography, reversed-phase chromatography, ion-exchange chromatography, and hydrophilic interaction liquid chromatography. Other techniques used to achieve separation of TCE and its metabolites include ion chromatography (IC), capillary electrophoresis (CE), and high-field asymmetric waveform ion mobility spectrometry (FAIMS). Methods for separation of TCE, DCA, and TCA in drinking water will also be discussed, due to the importance of drinking water as a source of

exposure to the compounds and because some of these methods may be adaptable for use with biological samples.

Gas Chromatography. TCE is a volatile organic compound (VOC). As would be expected, it is a good candidate for separation by GC. USEPA Method 551.1 is used for the analysis of TCE and several other VOCs in drinking water by GC with electron capture detection (ECD). This method has a minimum detection limit of 0.002 ng/mL for TCE from a 50-mL water sample (Munch and Hautman 1995). A minimum detection limit (MDL) of 0.02 ng/mL TCE was obtained with the GC-MS procedure described in USEPA method 524.2 (Munch 1995). Another GC-MS study was performed using USEPA method 524.2, in which drinking water was analyzed and an MDL of 5 ng/mL was determined for TCE (Eichelberger *et al*. 1990). GC-MS was also employed in the validation of a method for the quantitation of TCE in drinking water (Brown *et al*. 2003a). This method had an LOD of 1 ng/mL (3:1 signal:noise ratio) and an LLOQ of 5 ng/mL, determined as the lowest concentration sample which had less than 20% RSD and 20 % error over 3 days. TCE and 7 other VOCs, including chloroform and carbon tetrachloride, were measured with GC-MIMS (Bocchini *et al*. 1999). The method worked for all of the VOCs except TCE, which had a high variability in response, including a decrease in response when several TCE samples of the same concentration were injected. A method was developed for the analysis of PCE, TCE, cis-DCE, and trans-DCE using SPME (discussed in sample handling section) with GC and an FID detector (Xu *et al*. 1996). The detection limit for this method was 5 ng/mL for TCE.

DCA and TCA have pK_as of approximately 1.5 and 0.5, respectively (Urbansky 2000, Jia *et al* 2003, Sarzanini *et al*. 1999, Qu and Mou 1999). As a result, the two haloacetic acids are

found predominantly in their anionic form in solution. In order to run DCA and TCA by GC, the anions must be converted to a more volatile form. This is often accomplished either by derivitizing the compounds or by decreasing the pH to 0.5 in order to neutralize the anions. Numerous methods exist for the analysis of HAAs, including DCA and TCA, in drinking water. EPA methods 552.2 and 552.3 were developed for GC-ECD analysis of the 5 HAAs regulated by the USEPA (Domino *et al.* 2003). Minimum detection limits for Method 552.3 are 0.02 ng/mL for DCA and 0.019 ng/mL for TCA. Precision and accuracy data were also available for the methods for assorted drinking water samples (high ionic strength, high natural organic matter) and were within the ± 30% required by the agency. Xie (2001) used USEPA Method 552.2 with GC-MS rather than GC-ECD and obtained cleaner baselines with fewer interfering peaks. GC-MS (Berg *et al*. 2000, Sarríon *et al*. 1999, Sarríon *et al*. 2000, Wong *et al*. 2003) and GC-ECD (Williams *et al*. 1997, LeBel *et al*. 1997, Dalvi *et al*. 2000, Benanou *et al*. 1998, Singer *et al*. 1995, Krasner *et al*. 1989) were used to monitor the levels of HAAs in drinking water in the U.S., Canada, Switzerland, Saudi Arabia, France, and Spain. All methods successfully measured HAAs in drinking water, with limits of detection for DCA and TCA ranging from 0.01 - 4 ng/mL using GC-MS and 0.07 - 0.5 ng/mL using GC-ECD.

Several methods also exist for the analysis of TCE and its metabolites in biological samples. Merdink *et al.* (1998) dosed male B6C3F1 mice with TCE, CH, TCEOH, or TCA in order to determine levels of DCA formed as a metabolite from each of the above parent compounds. Rat blood samples were derivitized, so that DCA and TCA were converted to their methyl esters and LLE was performed. DCA, TCA, and CH were then analyzed by headspace GC-ECD. LODs reported using this method were 0.3 μM (49 ng/mL) and 1.4 μM (180.5 ng/mL) for TCA and DCA, respectively. The LOQs associated with this method were 0.7 μM

(65.4 ng/mL) for TCA and 1.9 μM (245 ng/mL) for DCA. LOD and LOQ values were not reported for CH. The authors did not detect DCA in the blood of mice dosed with any of the parent compounds.

Song and Ho (2003) performed a study in which male Sprague-Dawley rats were given 0.5 mg/kg silymarin (an anticancer and hepatoprotective compound) orally for 3 days prior to 2 days of oral dosing with 2.4 g/kg TCE. Urine samples were analyzed for TCEOH and TCA 6 days after dosing. An internal standard (DCA) and a mixture of 6:5:1 water:0.1 M sulfuric acid:methanol (for derivitization) were added to urine samples, and each sample was heated. The compounds were recovered by LLE in 1:1 hexane:dichloromethane and analyzed by GC-MS. LODs, as determined by a 3:1 S/N, were 3.4 ng/mL TCEOH and 4.6 ng/mL TCA. LOQs in this study were defined as the lowest concentration that had precision and accuracy values less than 20%. Values found for LOQs were 1.7 ng/mL for TCEOH and 2.3 ng/mL for TCA. Silymarin was found to alter the metabolism of TCE, as rats administered silymarin had increased formation of both TCA and TCEOH compared to rats dosed with only TCE. The authors conclude that this method can be used in future studies to determine the formation of metabolites of TCE in pharmacokinetic studies.

Forkert *et al.* (2003) measured levels of TCE, TCEOH, DCA, and TCA in the seminal fluid of eight infertile mechanics exposed to TCE occupationally. Semen samples (2 mL) were thawed at the time of analysis, and 1,3-dibromopropane (1 μL) was added as an internal standard. LLE was performed by adding 0.5 mL ethyl acetate to each sample. Samples were analyzed by headspace GC-ECD. TCE and TCEOH, respectively, were found in all of the workers over the following ranges: 10.2 – 2709.5 ng/mL and 1.35 - 12.75 ng/mL. TCA was found in one individual at a level of 2752 ng/mL and DCA was found in two individuals at

4719.5 and 6671 ng/mL. The authors also analyzed the urine of the same eight workers for TCA and TCEOH. Six workers had levels of TCA below 49 ng/mL. The remaining two workers had urinary TCA levels of 78.4 and 689.5 ng/mL. One worker had TCEOH present in his urine at 133.0 ng/mL, while the other workers had levels below 89.6 ng/mL.

Ashley *et al.* (1992, 1994) used a purge and trap concentrator in conjunction with GC-MS for the analysis of several VOCs (including TCE) in the blood of nonoccupationally exposed humans. Isotope dilution mass spectrometry was performed by adding 20 μL of stable isotope-labeled compounds to a 10-mL blood sample. A detection limit of 0.01 ng/mL was found for TCE in the more recent study. The mean blood TCE concentration was 0.039 ng/mL with a range of 0.016 – 0.061 ng/mL detected in 13% of individuals sampled. Recovery for TCE was 106 - 123% for the low, middle, and high concentration-spiked samples.

Brown *et al.* (2003b) developed a GC-MS method for the analysis TCE in the lung, liver, and kidneys of rats. TCE was extracted from each 100 μL sample by LLE with 200 μL of diethyl ether. Animals were dosed with 2 mg/kg TCE and samples were taken at 2, 5, 10, 30, 60, and 120 minutes post-dosing. The LOD for this method was 1 ng/mL and the LOQ was 5 ng/mL. The recovery for TCE from the lung, liver and kidney were 79.20 ± 10.8, 87.23 ± 2.78, and 79.93 ± 14.2, respectively. The method was validated by running 5 replicate samples at each of 3 QC concentrations. Precision and accuracy were measured as %RSD and % error and were below 15% for the two higher QC points and below 20% for the lowest QC point. A concentration vs. time profile was created from the data obtained in this study and closely matched a previous literature time profile curve for TCE in blood (Lee *et al*. 2000).

Muralidhara and Bruckner (1999) report a simple method for the determination of TCE, TCA, TCEOH, and DCA in rat lung, liver, kidney, and blood by headspace GC-ECD. A 6:5:1

solution of water: sulfuric acid: methanol is used to derivitize DCA and TCA to their methyl esters. The LOD for each compound in this study except DCA was 5 ng/mL. DCA had a LOD of 10 ng/mL. Percent recovery values for metabolites of TCE ranged from 52 - 100% in blood, 0 - 87% in liver, 15 - 86% in kidney, and 42 - 98% in lung at 7 different concentrations of the metabolites. Time-course studies in rats dosed with TCE indicated that over a 24-hour period, the amount of metabolites (particularly TCA) increased, peaking at 8 hours post-dosing.

Dehon *et al*. (2000) report a method for analyzing TCE, TCA, TCEOH, and PCE in a human fatality case. Levels of PCE, TCEOH, and TCA were measured from days 0-7 of hospitialization (patient died on day 7) and generally decreased over time. Several tissue samples were removed during the autopsy. TCE and PCE were measured in these tissue samples. The authors use SPME followed by GC-ECD and GC-MS for analysis. GC-ECD was used for quantitation, while GC-MS was performed in order to identify the compounds found in the peaks obtained from the GC-ECD runs. TCE was found to be in the highest concentration in the brain stem and cortex and in the lowest concentration in the liver and kidneys.

Yan *et al.* (1997, 1999) developed a GC-MS method for the analysis of CH, DCA, TCA, TCEOH, and MCA in human plasma. Samples were exposed to BF_3-MeOH for derivitization of anions to their methyl esters. Precision and accuracy values, expressed as %RSD and %Bias, respectively, were below 15% for all validation samples tested (concentrations ranged from 5 - 250 ng/mL). The total recovery (%) and LOQ (in ng/mL) for each compound in the most recent report are, respectively, as follows (reported as recovery, LOQ): CH 18.36 ± 0.97, 1295.1 ± 145.6; MCA 80.71 ± 4.20, 17.0 ± 1.9; DCA 86.84 ± 5.47, 15.5 ± 2.6; TCA 24.46 ± 2.21, 1248.3 ± 184.6; TCE 24.15 ± 1.08, 934.2 ± 69.6. Henderson *et al.* (1997) used the method to perform a study in which they found DCA to be a product of CH metabolism in children.

Kim *et al.* (1999) performed a study of TCA and DCA in human urine as an indicator of environmental exposure to the compounds. Samples were dervitized with sulfuric acid in methanol prior to GC-ECD analysis. The authors concluded that measuring TCA in urine was a valid biomarker of TCA exposure from household drinking water, while measuring DCA in urine did not give an accurate estimate of actual exposure to DCA in drinking water.

Ketcha *et al.* (1996) conducted a study in which blood samples containing DCA and TCA were derivitizated to their methyl esters using sulfuric acid in methanol and analyzed by GC-ECD. TCA was converted to DCA in the samples upon derivitization, resulting in artificially high DCA concentrations. The authors determined that the conversion occurred in the presence of sulfuric acid and reduced hemoglobin. Freezing blood samples overnight before adding the derivitizing reagent prevented the conversion of TCA to DCA. It was also noted that the use of lead acetate in the derivitization did not prevent the conversion of TCA to DCA.

Jia *et al.* (2003) developed a GC-MS method for the detection of TCA, DCA, and 7 other HAAs in water and human plasma and urine. GC-MS was carried out using an ECNCI (Electron Capture Negative Ion Chemical Ionization) source in the mass spectrometer. Precision and accuracy, in %CV and % error, respectively, were reported for the HAAs. Precision values for DCA and TCA were all below 20%, and accuracy for the two haloacetic acids ranged from 83-118%. Extraction recoveries for DCA and TCA ranged from 75-103%. LODs, as determined by a S/N of 5:1, were 0.05 ng/mL and 1 ng/mL for DCA and TCA, respectively.

Wu *et al.* (2002) report a SPME-GC-ECD method for the analysis of TCA, DCA, and several other haloacetic acids. Derivitization of the HAAs was carried out by adding sulfuric acid and methanol to each sample. The MDL in water was 0.6 ng/mL for both TCA and DCA.

Recoveries of 86-110% in urine and 82-110% in blood were found for TCA and DCA. Precision values of 1.1-14% RSD for DCA and 0.5-13% RSD for TCA were reported.

High Performance Liquid Chromatography. HPLC is used far less often than GC in the analysis of TCE and its metabolites. TCE is volatile and is therefore much better suited for GC analysis. DCA and TCA, however, would be expected to be better adapted to HPLC analysis than GC analysis due to their nonvolatile nature. The type of chromatography most commonly used with HPLC is reversed phase (RP) chromatography. RP HPLC columns (used with RP chromatography) contain nonpolar (most commonly C18) groups as the stationary phase. Therefore, nonpolar compounds are retained well. Small, charged polar molecules such as DCA and TCA, however, are not retained well. Retention of DCA and TCA on an HPLC column is possible, but considerable effort is involved in developing a chromatographic technique for these compounds. Challenges associated with retaining DCA and TCA by HPLC are partially responsible for there being fewer HPLC than GC methods for analysis of the compounds.

Kuklenyik *et al*. (2002) were able to retain TCA in urine samples using reversed phase chromatography by using a RP column containing polar embedded groups (Prism RP). The run was isocratic at 0.2 mL/min with a mobile phase consisting of 75% MeOH/25% 5 mM ammonium acetate (pH 5.2). An internal standard of isotopically labeled TCA (TCA-2-^{13}C) was used. All samples were extracted by SPE and run by LC-MS-MS. The LOD was determined by multiplying the standard deviation of responses of low concentration samples by 3 and was calculated to be 0.5 ng/mL. The LOQ was calculated similarly (by multiplying the standard deviation by 10) and was reported as 1.7 ng/mL. Accuracy of the method was reported as recoveries of 5 replicates each at 3 different concentrations. Recoveries reported at 1, 5, and 10

ng/mL were, respectively, 124%, 97%, and 98%. Precision was determined by taking the average of the CV of 21 measurements of QC solutions over an 8-week period and was calculated as 8.5%. TCA was measured in 402 urine samples from the Third National Health and Nutrition Examination Survey (NHANES III) and was found at detectable levels in 75.6% of the samples. This LC-MS-MS method was applied in another study in which the levels of TCA in the urine of individuals living in urban and rural areas were measured (Calafat *et al*. 2003). Individuals living in urban areas were found to have higher levels of TCA than those living in rural areas.

Carrero and Rusling (1999) also used reversed phase chromatography for the analysis of HAAs in drinking water by HPLC. The authors used an Alltech Econosphere C18 column and an electrochemical detector. The mobile phase used was acetate buffer (pH 5.5) containing 50 mM NaBr. Two sample preparation techniques, SPE and evaporation, were performed separately and compared to each other. With the evaporation method, 20 mL of water with 2 mL of added sodium biocarbonate are evaporated to 2 mL at 60°C. SPE samples are eluted from Sep-Pak cartridges with 2 mL of 12.5 µL/mL sulfuric acid. Estimated limits of detection for DCA and TCA were 4000 and 120 ng/mL, respectively, based on a S/N of 3:1 following a 100-µL injection. TCA was the only one of the six HAAs studied that was found in drinking water samples. Levels of TCA in drinking water from Windham Water Works in Windham, Connecticut were 155 ± 17 ng/mL and 168 ± 13 ng/mL using the SPE and evaporation techniques, respectively. The samples were also run by EPA Method 552.1 (GC-ECD). Using Method 552.1, TCA levels of 145 ± 14 ng/mL and 125 ± 20 ng/mL were found using SPE and evaporation, respectively.

Ion-pair chromatography employs a slightly different mechanism of retention than typical RP chromatography. With ion-pair chromatography, an ion-pairing agent, typically a charged organic compound (such as triethylamine or tetraethylammonium hydroxide), is added to the mobile phase. The organic portion of the molecule attaches to the stationary phase, and the charged portion of the compound is exposed. This allows polar and charged compounds to be retained by association with the exposed polar portion of the ion-pair agent. Loos and Barcelo (2001) used ion-pair liquid chromatography in order to retain several HAAs, including DCA and TCA. Sodium disulfite was added to all samples for preservation. Each sample was adjusted to pH 1.8 by addition of sulfuric acid prior to solid phase extraction. HAAs were subsequently detected by mass spectrometry. The LODs and LOQs reported in this paper were calculated from the response of blank samples. The LOD, LOQ, and linear range for DCA were, respectively, 0.8 ng/mL, 1.1 ng/mL, and 0.03 - 60 μg/mL. TCA had LOD, LOQ, and linear range values of 0.9 ng/mL, 1.3 ng/mL, and 0.1- 20 μg/mL. Recovery and precision values determined for DCA were 55% and 5 %RSD, respectively. TCA had recovery and precision values of, respectively, 75% and 5 %RSD. This method was used to measure the levels of several HAAs in drinking water samples at a drinking water treatment plant, in swimming pools, tap water, and river water. Low levels of both DCA and TCA (less than 16 ng/mL) were found in the influent, sandfilter, and effluent of the water treatment plant. No DCA was detected in any of the 3 swimming pools. However, the levels of TCA in swimming pools were 1700, 1500, and 1000 ng/mL. DCA was found at 35 ng/mL in tap water and 1-3 ng/mL in river water. TCA was present at 14 ng/mL in tap water and 4-308 ng/mL in river water.

Takino *et al*. (2000) also used ion-pair chromatography for the analysis of DCA and TCA in water samples. The suitability of three ion-pairing agents (N,N-Dimethyl-n-butylamine

[DMBA], tributylamine [TBA], and dibutylamine [DBA]) for the analysis of HAA9 was investigated. DMBA and TBA poorly retained some of the HAAs. Dibutylamine (DBA) was found to be best of the three ion-pairing agents. An acetonitrile-water mobile phase with 5 mM acetic acid and 5 mM DBA was used in this study. Isopropanol (IPA) was added post-column to improve ionization in the mass spectrometer. The column used was an Intersil ODS3 (5 μm, 150 x 2.1 mm). Water samples were run with no sample preconentration or cleanup steps (no LLE or SPE). Minimum quantitation limits (MQLs), defined as 10 times the standard deviation for a drinking water sample, were 0.024 ng/mL and 0.083 ng/mL for DCA and TCA, respectively. Interday and intraday precision values were, respectively, 1.5 %RSD and 5.9 %RSD for DCA and 4.9 %RSD and 6.6 %RSD for TCA. Correlation coefficient (R^2) values were greater than 0.999 for all 9 HAAs. Analysis of a water sample spiked with 1 ng/mL of each of HAA9 gave a response close to 1 ng/mL (1.07 ng/mL for DCA and 1.15 ng/mL for TCA).

Ion-exchange chromatography is similar to ion-pair chromatography in that a charged functional group is available for interaction with the analyte(s). However, with ion-exchange chromatography the charged group is present as part of the stationary phase of the HPLC column, thus eliminating the need for secondary interactions with ion-pairing agents. Narayanan *et al.* (1999) retained DCA, oxalic acid, glyoxylic acid, and glycolic acid in mouse plasma and urine samples using a Dionex AS11 ion exchange HPLC column with a conductivity detector. The mobile phase used was 0.01 mM NaOH in 40% MeOH, with a linear gradient from 0.01 mM to 60 mM NaOH in 40% MeOH over 30 minutes. Interday and intraday precision and accuracy for all four compounds monitored is reported as less than 1 %CV (n=10) in both plasma and urine. Mean recoveries were 100 ± 1.8% for all compounds in both plasma and urine. LODs were 50 ng/mL for all compounds in both plasma and urine. This method was applied to

measuring DCA and its metabolites in mice dosed with 300 mg/kg/day of DCA in drinking water. Samples were run by GC analysis to compare the GC and HPLC methods. The methods were comparable, as a correlation coefficient of 0.999 was observed between the two methods. Mice were given DCA-spiked drinking water for 28 days. The peak DCA concentration of 17.9 μg/mL occurred at 0 hrs post-dosing. No DCA was found in plasma 8 hours after ceasing administration of DCA. DCA was present at a level of 0.7 mg/mL in urine collected over a 24-hour period following a 26-day exposure to DCA in drinking water.

Hashimoto and Otsuki (1998) retained DCA, TCA, and seven other HAAs also by using ion-exchange chromatography. The column used in this study was a Supelcogel C-610H HPLC (crosslinked polystyrene resin) column, specifically designed for the separation of organic acids. A mass spectrometer was coupled to the HPLC for detection. The mobile phase used was 3% acetic acid in 20:80 ACN:H_2O. All samples analyzed were 200-mL water samples, which had 0.4 mL of 1 mg/mL 2,3 dichloropropionic acid, 0.3 g sodium thiosulfate, 80 g sodium sulfate, and sulfuric acid (to adjust to pH < 0.5) added to them. Each sample was then extracted twice with 10 mL MTBE. The final sample volume was 0.1 mL, resulting in a 2000-fold sample concentration. Within-day precision was calculated for all HAAs by injecting standard solutions five times in one day. These values were 445 ± 10 ng/mL for DCA and 63.2 ± 3.8 ng/mL for TCA (reported as mean ± standard deviation). Inter-day precision values, as calculated by injecting the standard solutions five times over two weeks, were 470 ± 11 ng/mL for DCA and 69.5 ± 4.9 ng/mL for TCA. Recoveries reported were, respectively, 89-98% for DCA and 80-89% for TCA in wastewater, river water, and seawater samples. LODs (after 2000-fold sample concentration) were 0.003 ng/mL for DCA and 0.070 ng/mL for TCA, as determined by a S/N of

3:1. DCA and TCA were found at levels lower than 2.28 ng/mL and 5.82 ng/mL, respectively, in all wastewater, river water, and seawater samples.

Hydrophilic interaction liquid chromatography (HILIC) is a type of chromatography in which the amount of water, not organic solvent, determines when analytes elute (more water = earlier elution). Column packings used with HILIC applications include silica, amino, and gel amide. HILIC-ion exchange chromatography, in which there is the additional retention mechanism of ion exchange, can also be performed. Increasing concentrations of a buffer containing an ion that competes with the analyte for charged sites on the column elicits elution. Dixon *et al*. (2004) used HILIC-ion exhange chromatography with tandem mass spectrometry for the analysis of DCA in drinking water. A Phenomenex Luna amino column was used in this study. The mobile phase consisted of ACN (A) and 40 mM ammonium formate (B). A gradient was performed with a total method run time of 15 minutes. Water samples (500 µL) were evaporated to dryness in a vacuum centrifuge and reconstituted in 100 µL of 60:40 ACN:H_2O. The LLOQ was 5 ng/mL and was determined as the lowest concentration at which precision (%RSD) and accuracy (% error) were less than or equal to 20%. The method was used to measure the level of DCA in several water samples. DCA was found below the regulatory level of 60 ng/mL in all samples and was found in lower quantities in homes using water filtration devices.

Capillary Electrophoresis. CE is another technique not as widely used as GC for the analysis of TCE metabolites. Only charged compounds (such as HAAs) can be measured by CE. The main advantage to CE is superior resolution (compared to GC and HPLC). In addition, HAAs measured by CE do not require derivitization. The main drawback when using CE is a high

LOQ. Ahrer and Buchberger (1999) developed a method for the analysis of 9 HAAs in water samples by CE-MS. New capillaries were conditioned with 0.5 M NaOH followed by water and prepared for permanent EOF reversal with 0.001% hexadimethrin bromide in methanol followed by flushing with methanol, followed by water, and finally CE buffer. Buffer was flushed through the capillary for 2 minutes between runs. Liquid-liquid extraction was performed in order to extract the HAAs from water samples. Sulfuric acid was added to 30-mL water samples until the pH was less than 0.5. Subsequently, 3 g copper sulfate and 12 g sodium sulfate were added, followed by the extraction solvent (MTBE). Two mL of the organic phase were then placed in a vial, 20 μL of water were added, and the sample was evaporated under nitrogen almost to dryness. The internal standards (5 ug/mL 3,5-dinitrobenzoic acid and maleic acid) are dissolved in 50 μL of methanol and added to the almost dry water sample. Larger sample volumes and amounts of sulfuric acid, copper sulfate, sodium sulfate, and MTBE added to samples are used when lower detection limits are needed. The linear range for DCA and TCA were 0.1 - 5 μg/mL and 0.2 - 3 μg/mL, respectively. Detection limits were determined by a 3:1 S/N ratio and were 0.1 μg/mL for DCA and 0.5 μg/mL for TCA. When analyzing 30-mL water samples (in which concentration steps are performed during and after LLE) detection limits were 0.3 ng/mL for DCA and 0.5 ng/mL for TCA. Recovery for DCA was 72% ± 8.2% and for TCA was 69% ± 4.5%. Analysis of DCA, but not TCA, was possible in real water samples, because high levels of bromide in the water favored the formation of brominated HAAs upon disinfection.

Kim *et al*. (2001) used capillary zone electrophoeresis with UV detection to analyze 5 HAAs in tap water. DCA was among the HAAs analyzed, but TCA could not be measured because of poor detection. Each 200-mL water sample was acidified to pH < 0.5 with sulfuric

acid. LLE was performed by adding 80 g of muffled sodium sulfate and 20 mL of MTBE to the water sample and shaking. The samples were re-extracted with another 10 mL of MTBE. Samples were dried to 50 µL in a rotary evaporator and to dryness under nitrogen. One-hundred µL of electrolyte were then added to each sample prior to CZE analysis. The capillary was pretreated with 0.1 M NaOH, followed by deionized water, and finally the running buffer. The carrier electrolyte used was 25 mM phosphate and 0.5 mM cetyltrimethylammonium chloride (CTAC). Recovery for DCA was 95.8% ± 9.1%, and the precision for recovery was 9.5% RSD (n = 3). DCA was found in tap water at a level of 5.7 ± 0.5 ng/mL (n = 3).

Martinez *et al*. (1998b) also used CZE with indirect UV detection for the analysis of HAAs, including DCA and TCA, in water. LLE was performed in this method similarly to Ahrer and Buchberger (1999) in that a 30-mL sample was adjusted to pH 0.5, and then 12 g sodium sulfate, 3 g of copper sulfate, and 3 mL MTBE were added to the sample. Each sample was evaporated to near dryness under nitrogen, and then 100 µL of deionized water was added. Two different electrolytes were used and compared to each other for the analysis of HAAs. The two electrolytes were potassium hydrogenphthalate and 2,6-napthalenedicarboxylic acid dipotassium (NDC). NDC was found to have better sensitivity and selectivity than hydrogenphthalate for measuring HAAs. Therefore, a 4 mM NDC and 0.5 mM CTAB (hexadecyltrimethylammonium bromide) run buffer at pH 7.5 was used for the study. The capillary was rinsed with a background electrolyte solution before each run, and the UV detector was set at 235 nm. LODs were determined by a S/N of 3:1 and were 0.15 µg/mL and 0.50 µg/mL (n = 10 for each) for DCA and TCA, respectively. Precision, measured as %RSD, was determined as 1.5 for DCA and 1.9 for TCA (n = 10 for each). Recovery values were 70% for DCA and 80% for TCA. Tap water samples from two different Spanish cities were analyzed for

DCA and were found to contain levels of 8 ng/mL and 18 ng/mL DCA. TCA was also present in these samples, but was below the quantiation limit and not reported.

Martínez *et al*. (1998a) performed another study in which four types of SPE cartridges were used to extract HAAs from tap water prior to CE analysis. Water samples were tested before and after chlorination and at several points in the water supply. The four types of SPE tubes investigated were LC-SAX (quaternary ammonium anion exchange), LiChrolut EN (highly crosslinked styrene-divinylbenzene), Envi-Carb (graphitized carbon black), and Oasis HLB (macroporous polydivinylbenzene-co-N-vinylpyrrolidone copolymer). LiChrolut EN SPE cartridges were determined to work best for the extraction of HAAs from drinking water. Samples were acidified to pH 0.5 prior to SPE. Steps in the SPE were as follows: 1) LiChrolut EN SPE cartridges were conditioned with MeOH, 2) the sample was loaded, 3) Milli-Q water was added as a wash step, and 4) the HAAs were then eluted with 50:50 MeOH:water and filtered prior to CE analysis. Real water samples were also run by LLE-GC for comparison to the CE method. Recovery was assessed at 5 different concentrations and was 82-104% for DCA and 85-101% for TCA. The linear range was 5-80 ng/mL for both DCA and TCA, and both compounds had a LOD of 2 ng/mL. Precision, determined as %RSD, was 5.5 for DCA and 6.7 for TCA. Samples of water taken before the disinfection process showed no detectable amounts of HAAs when run both by CE and GC-MS. DCA (at levels of 7.6 - 9.3 ng/mL) and TCA (at levels of 9.8 - 17.4 ng/mL) could be detected by CE after chlorination and in increasing amounts with increasing distance from the water treatment plant. GC-MS analysis showed similar results, with the only difference being that the samples run by GC-MS resulted in about a 3 ng/mL lower response than the corresponding sample run by CE.

The same authors performed another study in which four different electrolyte systems, on-line preconcentration, and reversal of EOF are investigated (Martínez *et al*. 1999). Among the electrolyte systems used in this study are: 1) 12 mM phthalate (pH = 6), 2) 4 mM NDC (pH = 7.5), 3) 20 mM borate (pH = 9.6), and 4) 10 mM chromate (pH = 8.7). The best electrolyte system was 4 mM NDC. Adding a surfactant, 0.5 mM CTAB, to reverse the EOF increased the resolution between the HAAs (particularly TCA and DBA) and gave good LODs and correlation coefficients. Electrokinetic injection was found to work well for sample preconcentration. However, due to the matrix effects present in the swimming pool water samples, SPE still had to be performed prior to sample analysis, and the SPE procedure was modified from the procedure described in a previous publication (Martínez *et al*. 1998a). Twenty-five mL of swimming pool water were acidified to pH 0.5 with sulfuric acid, passed through a SPE cartridge, rinsed with 0.5 mL deionized water, and eluted with 1 mL of methanol. In addition, it was necessary to dilute the eluent 1:3 with deionized water prior to injection. The linear range for both DCA and TCA was 40-160 ng/mL, with an r^2 value of > 0.99. Precision, determined as %RSD, was 6.6 for DCA and 10.2 for TCA. Recovery values for DCA and TCA were, respectively, 60% and 58%. LODs for HAAs were not reported in this study. The levels of DCA and TCA found in swimming pool water were, respectively, 68.8 ng/mL and 42.1 ng/mL.

Ion Chromatography. Ion chromatography is a separation technique similar to HPLC. IC is used more frequently with ionic analytes, and conductivity detectors are most commonly used with this type of chromatography. Sarzanini *et al*. (1999) developed and compared IC methods for the determination HAA5 in drinking water. The two types of retention mechanisms tested were ion-interaction and anion-exchange chromatography. Sample preconcentration was

accomplished using LiChrolut-EN SPE cartridges conditioned with 3 mL methanol and 3 mL high purity water prior to sample loading. Detection limits for DCA and TCA were, respectively, 50 ng/mL and 150 ng/mL using the ion-interaction chromatography with a 50% methanol, 3.5 mM CTAC, pH 5.0 mobile phase. Recoveries of DCA and TCA using this method were > 99%. However, the optimum conditions for ion-chromatography separations of HAAs were with the ion-exchange method coupled and are as follows: eluent of 35% acetonitrile, 18 mM NH_4Cl, and 10 mM NaCl used with UV detection and sample preconcentration with LiChrolut-EN SPE cartridges. Under these conditions, quantitative limits (defined as 3:1 S/N) are 7 ng/mL for DCA and 10 ng/mL for TCA. Recoveries for DCA and TCA are, respectively, 83.7 ± 13 and 81.8 ± 11.

Qu and Mou (1999) determined levels of MCA, DCA, and acetic acid in various steps in the production of MCA with an IC method coupled with a conductivity detector. Anion exchange was the retention mechanism used with an eluent of 2.5 mM NaOH and 10% MeOH. DCA had good linearity in the range of 0.15 – 20 μg/mL, with a correlation coefficient of 0.9998. A detection limit of 25 ng/mL, defined as 3:1 S/N, was achieved for DCA using this method. Recovery of DCA was 94.6-99.6%. Using this method, the authors were able determine that their batches of MCA were no longer contaminated with DCA after making improvements in their process for making MCA.

Ko *et al*. (2000) developed a method for the analysis of DCA and TCA in drinking water using an ion chromatograph and electrochemical detector. A 200-mL aqueous sample was acidified to pH < 0.5 with HCl and extracted with two 15-mL portions of MTBE. Ten mL of the extract (MTBE) were then extracted twice with 1 mL of water, and the water extract was injected into the ion chromatograph. An IonPac AS11 analytical column was used with an eluent of 5.0

mM NaOH and reagent water with the following gradient: 10% A from 0-9 min and 100% A from 15-30 min. MDLs were determined by multiplying 3.14 times the standard deviation of seven replicates of a sample approximately 2 to 5 times the S/N ratio of the instrument. The MDLs for DCA and TCA, respectively, were 0.45 ng/mL and 1.5 ng/mL. Recoveries for DCA were 90-96% ± 6 to 9% and for TCA were 95-108% ± 4 to 10%. This method allowed for tracking of DCA and TCA formation after ozonation and chlorination of drinking water and revealed that ozonation affects the levels of DCA and TCA in some, but not all, water reservoirs.

High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS). FAIMS is a relatively new technique for separating ions and introducing them into a mass spectrometer. Ions are separated in FAIMS by changing ion mobility in the presence of a high electric field. A FAIMS method for the analysis of HAA9 in water was developed by Ells *et al*. (2000). All HAA solutions for analysis were prepared in a buffer consisting of 9:1 methanol:deionized water containing 0.2 mM of ammonium acetate. Correlation coefficients of 0.9939 and 0.9936 were found for calibration curves of DCA and TCA, respectively. Detection limits for DCA and TCA, as determined by using 3 times the standard deviation of the background, were 10 pg/mL and 36 pg/mL, respectively. In addition, a comparison between ESI-FAIMS-MS and traditional ESI-MS was made. The authors found that FAIMS-MS had detection limits four orders of magnitude lower than ESI-MS for the HAA tested (BDCA). Gabryelski *et al*. (2003) compared ESI-FAIMS-MS to GC and GC-MS methods for the detection of HAA9. In general, the methods were comparable, with FAIMS having the advantage of very little sample preparation compared to GC methods. LOQs (defined as 5 times the standard deviation of the blank) of 0.55 ng/mL for DCA and 0.60 ng/mL for TCA were obtained in samples run by ESI-FAIMS-MS.

DETECTORS

Overview. Several types of detectors are available to be coupled with the separation techniques discussed in this paper. Some of these include mass spectrometers (MS), electron capture detectors (ECD), flame ionization detectors (FID), ultraviolet detectors (UV), and conductivity detectors. Mass spectrometers are commonly used with GC and HPLC and less frequently with CE. FID and ECD detectors are used almost exclusively with gas chromatography. UV detectors are commonly used in conjunction with HPLC or CE analysis. Conductivity detectors are used with most IC and some HPLC applications.

Mass Spectrometers. Mass spectrometers are commonly used as detectors for GC and LC applications. Their main advantages are a high specificity and lower limits of detection due to their ability to discriminate against noise. Mass spectrometers have the ability to monitor only the mass (or transition from one mass to another by fragmentation) of the analyte(s) of interest. This means that all other peaks that would typically be observed in the chromatogram and potentially interfere with analyte peaks are not present in chromatograms (providing that other analytes are of a different mass). The result is that the noise is lower, resulting in a higher S/N and, therefore, lower LOQs. An additional advantage of mass spectrometers is that each peak of interest can often be identified using a full-scan mass spectrum. Therefore, one can definitively know the identity of the peak(s) in a chromatogram. There are several different methods of separating compounds based on their mass. As a result, there are several different types of mass spectrometers, each using a different mass analyzer (method of separating compounds based on

mass). Each different type of mass analyzer that has been used for the detection of TCE and/or its metabolites will be discussed in this section.

Quadrupole mass spectrometers are the most commonly used mass spectrometers in quantitative analysis. Advantages of the quadrupole mass spectrometer include their low maintenance requirements and ruggedness. Quadrupole mass spectrometers are also easily interfaced with GCs and LCs (and less easily with CEs), making it possible to analyze a wide array of compounds. The main disadvantage of quadrupole mass spectrometers is poor mass resolution (typically 1 Da). This, however, is more of a drawback when analyzing compounds with larger molecular weights and multiply charged compounds (such as proteins and peptides) and is not typically a setback with small molecules. The most commonly used means of sample introduction into quadrupole mass spectrometers, with liquid samples, is electrospray ionization (ESI). With this method of sample introduction, the liquid is introduced through a charged capillary. The analyte molecules are charged (positively or negatively depending on the ionization mode) and desolvated by spraying under potential through drying gases. Single quadrupole mass spectrometers can obtain the mass spectrum of analytes injected (MS analysis). When coupled with chromatography and run in SIM mode, single quadrupole instruments yield chromatograms containing peaks that are only of the mass(es) of interest. Triple quadrupole mass spectrometers have the added advantage using the first quadrupole mass analyzer to select an analyte of interest and transmit it to the second quadrupole where it is dissociated following collision with neutral gas molecules. This collision process results in the formation of fragment ions that are transmitted to the third quadrupole (MS-MS analysis). The selection of specific analyte ion(s) with the first quadrupole and diagnostic abundant fragment ion(s) with the third quadrupole results in a technique called multiple reaction monitoring (MRM). When used in

conjunction with a separation technique and run in MRM mode, triple quadrupole mass spectrometers generate chromatograms that only contain peaks for compounds that have undergone a designated mass transition (that is, the compound has been fragmented from a known precursor compound m/z to a known fragment m/z).

A few methods exist for the analysis of HAAs with direct injection into a triple quadrupole mass spectrometer (no separation technique used). Brashear *et al*. (1997) injected samples directly (after sample cleanup) into a triple quadrupole mass spectrometer for the analysis of MCA, DCA, and TCA in blood samples from human volunteers exposed to 100 ppm of TCE via inhalation for 4 hours. Blood samples were collected during exposure and post exposure up to 94 hours. All samples were centrifuged to obtain plasma, and 0.5 mL aliquots of plasma were combined with 0.1 mL of water. Each sample was acidified with 0.5 mL of 10% sulfuric acid and extracted into 2.5 mL of diethyl ether. The extract was then frozen at -20°C for one hour, thawed, centrifuged at 4000 x *g* for 45 minutes, and evaporated under nitrogen. Each sample was reconstituted in 0.5 mL of 75:24:1 methanol: water: acetic acid and centrifuged at 2000 x *g* for 20 minutes. Negative electrospray ionization and tandem mass spectrometry (MS-MS) in MRM (SRM) mode was used for the analysis of all HAAs. The following mass transitions were monitored for HAA analysis: 127 → 83 for DCA, 161 → 117 for TCA, and 153 → 93 for MCA. These transitions corresponded to the loss of 44 (CO_2) for DCA and TCA and 60 (acetic acid) for MCA. Calibration curves were found to be linear for the HAAs up to 100 ng/mL. LODs, as determined by a 3:1 S/N, were 4 ng/mL for both DCA and TCA. Extraction efficiency for all HAAs from plasma was 75%. Precision and accuracy values were determined for all HAAs at low (10 ng/mL), medium (50 ng/mL) and high (100 ng/mL) concentrations. Accuracy was determined as average extraction efficency and precision was computed as %CV.

Values found for accuracy were as follows: 86 - 105% (TCA, intraday), 89 - 94% (TCA, interday), 96-101% (DCA, intraday), and 81 - 103% (DCA, interday). Numbers for precision were as follows: 31-58 %CV (TCA, intraday), 28-63 %CV (TCA, interday), 25-34 %CV (DCA, intraday), and 31-42 %CV (DCA, interday). Low levels (1-6 ng/mL) of DCA were found in individuals exposed to TCE, and the levels of DCA dropped shortly after TCE exposure. TCA levels in the blood increased and eventually leveled off 8 hours after exposure to TCE and were found as high as 10-12 μg/mL. Blood levels of TCA did not decrease from 8 hours post-dosing until the last sample was taken at 94 hours post-dosing.

Magnuson and Kelty (2000) also describe a method for the analysis of HAAs by directly injecting samples into a triple quadrupole mass spectrometer. This method, however, is for HAA9 in water samples and requires the complexation of HAAs to perfluoroheptanoic acid. Water samples (188 mL) were acidified with 20 mL of 50% sulfuric acid. Sodium sulfate (58 g) and copper sulfate pentahydrate (7 g) were added to each sample. MTBE (3 mL) was added, and after 5 minutes the MTBE layer was removed. A 500-μL portion of the extract was placed into a vial with perfluoroheptanoic acid, and 30 μL of this solution was injected into the mass spectrometer. The ionization technique used was ESI negative, and all HAAs were monitored on Q3 by scanning the mass range of the fragmentation products. Mass-to-charge ratios observed for the DCA and TCA complexes were 491 and 525, respectively. MDLs, determined as 3.14 times the standard deviation of 7 replicate injections of solutions 3-6 times the concentration of the MDL, were 0.32 ng/mL for DCA and 0.13 ng/mL for TCA. A comparison showed that blank tap water and deionized water samples spiked with equal concentrations of HAAs had comparable responses, meaning matrix effects among these types of water samples were not an issue. However, concentrations of DCA and TCA were, respectively, approximately 5 and 14

times higher when fortified water samples were chlorinated with increasing chlorine concentrations (chlorine to carbon mole ratio of 0.8).

Several methods using quadrupole mass spectrometers have been previously described in the separations section of this paper. An interesting observation was made in the study by Ells *et al*. (2000), in which a triple quadrupole instrument was used. All of the monochlorinated and dichlorinated HAAs were found to fragment when using pure nitrogen as the carrier gas. However, in order to detect trihaloacetic acids, it was necessary to add small amounts of CO_2 with the nitrogen. Also interesting to note is that Xie (2001) was able to obtain cleaner chromatograms with fewer interfering peaks and lower MDLs for HAAs using EPA Method 552.2 with a single quadrupole mass spectrometer instead of an ECD. Single quadrupole mass spectrometers are predominantly used with GC methods and have been utilized for the GC-MS analysis of TCE in urine (Song and Ho 2003) and tissues (Dehon *et al*. 2000), DCA in plasma (Yan *et al*. 1997), and CH in plasma (Yan *et al*. 1999, Henderson *et al*. 1997). In addition, HPLC (Takino *et al*. 2000, Hashimoto and Otsuki 1998, Loos and Barcelo 2001) and CE methods (Ahrer and Buchberger 1999) coupled with single quadrupole mass spectrometers also exist for the analysis of HAAs in water samples. Triple quadrupole mass spectrometers are most commonly used with HPLC methods. LC-MS-MS methods for the analysis of TCA in human urine (Kuklenyik *et al*. 2002, Calafat *et al*. 2003, Ells *et al*. 2000) and HAAs in water (Hashimoto and Otsuki 1998) have been previously developed and were discussed in an earlier section of this paper.

Magnetic sector mass spectrometers are a second class of MS instruments (utilizing a different type of mass analyzer). The main advantage of this type of mass spectrometer is the ability to perform high resolution analysis, meaning that this type of mass spectrometer can

distinguish the difference between two compounds that are very close to one another in mass (up to 4 or more decimal places). This feature also allows exact mass measurements to be obtained for compound identification purposes. The high resolution capability of magnetic sectors is used to discriminate against chemical noise, and therefore these instruments can be quite sensitive. Disadvantages of this type of instrument include higher routine maintenance requirements, low sample throughput, and high expense associated with replacing parts and electronics relative to other mass spectrometers. Magnetic sector instruments are almost exclusively coupled with GCs. However, more modern instruments do possess the ability to interface with a HPLC (high resolution electrospray). All of the studies utilizing magnetic sector instruments mentioned in this paper are interfaced with GCs and used for the analysis of TCE (Ashley *et al*. 1992, Ashley *et al*.1994, Brown *et al*. 2003a, Brown *et al*. 2003b).

Time of flight (TOF) mass spectrometers, like magnetic sector instruments, have the advantages of high resolution and exact mass measurement capabilities. Limitations to TOF instruments include higher maintenance costs than quadrupole mass spectrometers and limited dynamic range. TOF instruments have recently been produced with a linear dynamic range of 3–4 orders of magnitude. However, it is unclear how this will translate into quantitative bioanalytical studies. TOF mass spectrometers are commonly interfaced with GCs and LCs. However, the only method utilizing a TOF mass spectrometer that was found in the literature (Debré *et al*. 2000) was with direct injection of samples into the mass spectrometer (no separation technique used). The study was designed for the analysis of HAA9 in water. Initial work was performed on a CE coupled with a single quadrupole mass spectrometer. Data were then collected on the TOF instrument and compared to the CE-MS data. The authors found the TOF to be suitable for the analysis of HAAs in drinking water and concluded that although the

detection limits (not given) were higher than desired, in the near future new instrumentation would likely make it possible to reach target detection limits.

Ion trap mass spectrometers have the ability to perform MS^n analysis, meaning that the instrument can fragment a compound, then fragment the fragment ion(s) to obtain a spectrum from these ions, and then can choose one or more ions from this spectrum and further fragment them and so on. MS^n capability is the main advantage of ion trap mass spectrometers and is most advantageous when performing structure elucidation studies (that is, compound identification). A disadvantage of ion trap mass spectrometers is that while it is good for qualitative work, it is does not work as well for quantitative studies. The reason for this is a phenomenon known as the space-charge effect. Ions in an ion trap mass spectrometer are fragmented and trapped in a doughnut-shaped area between ring electrodes and endcaps. When ions are fragmented several times, more and more ions are trapped in a small area. Too much charge begins accumulating in a small space. As a result, calibration curves do not have proportionally increased responses at higher concentrations, as is the case in the lower and middle portions of calibration curves (i.e. the calibration curve begins to flatten off at the high end). Newer ion trap instruments known as linear ion traps have a different source design that circumvents these problems. Ion trap instruments are commonly interfaced with both GCs and HPLCs. All of the methods using ion trap mass spectrometers described in this paper are coupled to GCs. These methods were developed for the analysis of TCE in drinking water (Eichelberger *et al*. 1990, Bocchini *et al*. 1999, Sarríon *et al*. 1999), HAAs in water (Sarríon *et al*. 1999, Sarríon *et al*. 2000), and HAAs in plasma, urine, and water (Jia *et al*. 2003).

Electron Capture Detection. Electron capture detectors (ECDs) are commonly used with GC methods. Samples subjected to an ECD pass over a radioactive β-emitting substance (usually nickel-63) that causes ionization of the carrier gas and production of electrons. The ECD is specific for detecting compounds containing electronegative groups, making it very sensitive for analysis of halogenated compounds such as TCE, DCA, TCA, and CH. In many cases, the ECD detector is the most sensitive detector for the analysis of halogenated compounds. However, the lower level of noise provided by methods using mass spectrometers as the detector often results in LODs similar to those with ECD detectors. Nonetheless, ECD detectors are very useful in the detection of TCE and its metabolites. The USEPA uses GC-ECD for the analysis of TCE (Munch and Hautman 1995, Munch 1995), DCA, and TCA (Domino *et al*. 2003). In addition, the majority of the GC methods discussed in this paper employ ECD detection. These methods include the simultaneous analysis of TCE and its metabolites in several biological matrices, including lung, liver, kidney, and blood (Merdink *et al*. 1998, Muralidhara and Bruckner 1999). A GC-ECD method also exists for the analysis of TCE in seminal fluids of humans exposed to TCE occupationally (Forkert *et al*. 2002). There are also several GC-ECD methods for the analysis of HAAs in water (Williams *et al*. 1997, LeBel *et al*. 1997, Dalvi *et al*. 2000, Benanou *et al*. 1998, Singer *et al*. 1995, Krasner *et al*. 1989) and biological samples (Kim *et al*. 1999, Wu *et al*. 2002, Ketcha *et al*. 1996).

Flame Ionization Detection. Flame ionization detection (FID) is another type of detection commonly used with GC analysis. With an FID detector, hydrogen and air are combined and ignited electrically. Organic compounds are ionized in the flame, producing electrons that are measured as the signal, or response. There is no feature that makes FID particularly useful for

the analysis of TCE and its metabolites as compared to other possible analytes. This is demonstrated by the fact that only one method discussed in this paper used an FID detector (Xu *et al*. 1996). The single GC-FID study found in the literature was for the analysis of TCE and two other chloroethenes in aqueous samples. Results reported for the LOD (5 ng/mL) were comparable to several GC-MS and GC-ECD methods.

Ultraviolet Detection. Ultraviolet (UV) detection is used most commonly with HPLCs and CEs. Compounds are detected with UV detection as absorbance of UV radiation. A wavelength of UV light at which the analyte most strongly absorbs is chosen for the detector. Conjugated compounds (those with alternating double and single bonds) are the strongest UV absorbers. TCE and its metabolites have one double bond and therefore absorb UV light, but are not ideal candidates for methods employing UV detection, unless they are derivitized with a significant chromophore. Poor UV absorption and high detection limits are likely the cause for the scarcity of HPLC-UV methods in the literature. Generally speaking, ECD and MS are preferred over UV detection for the detection of TCE and its metabolites, due to their ability to achieve lower LODs. One CE-UV method (Kim *et al*. 2001) and one IC-UV method (Sarzanini *et al*. 1999) for the analysis of HAAs are discussed in this paper. CE methods in general have high LODs compared to other sample separation techniques. Therefore, the weak UV absorption of the HAAs is less noticeable with CE methods, because it is difficult to achieve low LODs to begin with. Lower LODs can be achieved with CE methods using a UV detector either by concentrating samples (Kim *et al*. 2001, Martínez 1998a, Martínez *et al*. 1999) or by using indirect UV detection. Indirect UV detection involves adding a chromophore to the mobile phase in order to saturate the UV detector. Analytes are then detected as a drop in UV

absorbance. Three CE-indirect UV methods are discussed in this paper (Martínez 1998a, Martínez 1998b, Martínez *et al*. 1999).

Conductivity Detectors. Conductivity detectors are commonly used with IC and HPLC methods. This type of detector is particularly useful when the analytes of interest are ions. Conductivity detectors are generally rugged and inexpensive compared to other detectors. One drawback with this type of detector, however, is that it can become swamped by mobile phase ions. Ion chromatography is a particularly good separation technique to couple with conductivity, because this technique separates ionic compounds. Similarly, many studies using an HPLC fitted with a conductivity detector can be found in the literature, because HPLC is a good separation technique for water-soluble analytes (including ions). Two ion chromatography methods for the analysis of HAAs in water (Qu and Mou 1999, Ko *et al*. 2000) and one HPLC method using a conductivity detector for the analysis of DCA in plasma and urine (Narayanan *et al*. 1999) are discussed in this review.

EXTRACTION TECHNIQUES

Overview. Before analyzing TCE and its metabolites, it is often necessary to extract the compound(s) from the matrix in which they are found. There are several methods for extracting these compounds from various matrices. Some extraction techniques available include liquid-liquid extraction (LLE), solid phase microextraction (SPME), protein precipitation (PP), and solid phase extraction (SPE). Typically, each of these means of extraction works best in certain

situations. How each type of extraction works and in which situation each works best will be discussed in this section.

Liquid-liquid extraction. LLE is a traditional means of extracting compounds in which two immiscible solvents, typically an organic and an aqueous solvent, are placed in the same container along with the analyte of interest. Ideally, the analyte of interest is either very hydrophilic or very hydrophobic, and therefore partitions almost completely into one of the two layers. The layer in which the analyte is found is then collected. This extraction technique is particularly useful for TCE, a lipophilic compound found in drinking water. An organic solvent is simply added to a drinking water sample, and TCE partitions into the organic phase, which is collected (Brown *et* al. 2003a). Although the HAAs are very water soluble, many methods exist in which HAAs in drinking water are either derivitized or neutralized by acidification in order to make them more lipophilic and volatile prior to LLE extraction (Ko *et al*. 2000, Ahrer and Buchberger 1999, Martinez *et al*. 1998b, Kim *et al.* 2001, Domino *et al*. 2003, Xie 2001, Kim *et al*. 1999, Dalvi *et al*. 2000, Williams *et al*. 1997, LeBel *et al.* 1997, Wong *et al*. 2003, Hashimoto and Otsuki 1998, Magnuson and Kelty 2000, Wu *et al*. 2002, Gabryelski *et al*. 2003). In addition, LLE can also be used to extract TCE (Brown *et al*. 2003b, Song and Ho 2003), CH (Yan *et al*. 1999, Henderson *et al*. 1997), and DCA and TCA (Yan *et al*. 1997, Jia *et al*. 2003, Merdink *et al*. 1998, Ketcha *et al*. 1996, Brashear *et al*. 1997) from biological matrices by extracting the organic layer after adding an organic solvent to tissue homogenate, plasma, or blood samples.

Solid Phase Microextraction. SPME is a relatively new extraction technique developed by Janusz Pawliszyn in the 1980s. With this technique, a SPME fiber is inserted either into the sample headspace (air above the liquid in a sample) or immersed in the sample (Pawliszyn 2000). The SPME fiber has a chemical coating on the outside, which is similar to the coating on the inside of GC columns. A suitable analyte has a chemical affinity for the coating on the SPME fiber. As a result, the analyte adsorbs to and becomes concentrated on the fiber. Desorption of the analyte is most often accomplished thermally in the injection port of a GC.

Care must be taken when using SPME for several reasons. Proteins in blood and tissue samples can irreversibly bind to the SPME fiber, thereby decreasing its ability to adsorb analytes. Therefore, most methods place the SPME fiber in the sample headspace to mimimize this interaction. SPME fibers with different types and thicknesses of coatings are available for purchase. It is important to choose the type of coating that has a strong (but not irreversible) affinity for the analyte of interest. In addition, the fiber must be used with compatible solvents. Polydimethylsiloxane (PDMS) fibers, for example, must be used almost exclusively with water. Most organic solvents will eat the coating off of the SPME fiber, rendering it useless for subsequent extractions. Also, with SPME several parameters are analyte-specific and need to be optimized for each analyte studied. Some of these parameters include: length of time to leave the sample in the fiber headspace, temperature at which to heat sample (in order to drive volatile analytes into the headspace), length of time to heat the sample, and amount of salt to add to samples (again, to drive volatile analytes into the headspace).

Several methods using SPME are discussed in this paper for the analysis of TCE in biological samples (Dehon *et* al. 2000, Xu *et al*. 1996, Dixon *et al*. 2004), HAAs in drinking water (Sarrión *et al*. 1999, Sarrión *et al*. 2000, Wu *et al*. 2002, Gabryelski *et al*. 2003), and

HAAs in biological samples (Dehon *et* al. 2000). PDMS fibers were used for each TCE study (Dehon *et* al. 2000, Xu *et al*. 1996, Dixon *et al*. 2004) and three of the HAA studies (Sarrión *et al*. 1999, Wu *et al*. 2002, Gabryelski *et al*. 2003). In another HAA study using SPME fibers, five types of SPME fibers were tested (Sarrión *et al*. 2000). The 5 types of fibers (and each fiber thickness) tested were PDMS (polydimethylsiloxane, 100 μm), PA (polyacrylite, 85 μm), CAR-PDMS (carboxen-polydimethylsiloxane, 75 μm), PDMS-DVB (polydimethylsiloxane-divinylbenzene, 65 μm), DVB-CAR-PDMS (StableFlex divinylbenzene-carboxen-polydimethylsiloxane, 50/30 μm). The CAR-PDMS had the highest extraction efficiency for DCA, TCA, and all of the other HAAs analyzed. Several of the SPME methods discussed optimization of the SPME for a particular analyte(s) (Xu *et* al. 1996, Sarrión *et al*. 2000, Wu *et al*. 2002, Dixon *et al*. 2004), and a SPME theory paper (Pawliszyn 2000) describes the in-depth details of SPME.

Solid Phase Extraction. SPE is an isolation technique in which the analyte is (usually) retained on a cartridge, while unwanted chemical entities in the sample matrix either pass through or are irreversibly bound to the cartridge. The analyte is subsequently eluted off of the cartridge and is commonly evaporated to dryness and reconstituted. SPE cartridges are essentially small, disposable HPLC columns. Several different sorbents (packing material to which analyte adsorbs) and cartridge sizes are available with SPE. Sorbents available include C18, C8, C2, phenyl, amino, WAX, SAX, WCX, and SCX. Cartridge size is generally determined by the mass of the sorbent (packing material, in grams), and the cartridge is usually in a tube with a volume (in mL) of 1 - 10 times the mass of the cartridge. Like SPME, SPE also involves optimization of the extraction method. SPE, in general, has 5 steps involved and each of these

steps needs to be optimized. The 5 steps include 1) conditioning the cartridge, 2) equilibrating the cartridge, 3) loading the sample, 4) washing the cartridge, and 5) eluting the analyte. SPE is used primarily for nonvolatile, lipophilic compounds with polar groups (e.g., pesticides and pharmaceuticals). Volatile compounds are, in general, not good candidates for SPE because they are exposed to the air for too long. As a result, no methods for the extraction of TCE by SPE were found. Several methods do exist, however, for the analysis of HAAs in water (Benanou *et al*. 1998, Martínez *et al*. 1999, Martínez *et al*. 1998a, Martínez *et al*. 1998b, Loos and Barcelo 2001, Carrero and Rusling 1999, Sarzanini *et al*. 1999) and urine (Kuklenyik *et al*. 2002, Calafat *et al*. 2003) using SPE.

Protein precipitation. PP is a type of extraction in which either a solvent (typically ACN) or an acid (typically perchloric acid) is added to a sample to denature proteins. The precipitating reagent is usually kept ice-cold because ice-cold liquids usually yield a more complete precipitation. Denatured proteins settle to the bottom, leaving the remaining sample a transparent straw yellow or clear color. Typically, a volume of PP reagent equal to 2-3 times the sample volume is added to each sample. Samples are then centrifuged in order to compact the denatured proteins into a pellet, maximizing the amount of liquid that can be removed. Depending on the sensitivity of the instrument and the compound and matrix involved, PP may be performed alone or in conjunction with another extraction technique. For example, PP is commonly performed prior to SPE. One method for the analysis of DCA (and its metabolites) discussed in this paper uses perchloric acid for protein precipitation of blood samples prior to HPLC analysis (Narayanan *et al*. 1999).

PRESERVATION OF SAMPLE SPECIATION

Overview. When analyzing for TCE and its metabolites, it is extremely important to take precautions with sample handling and preparation to ensure that the analytes do not change from one chemical entity to another (i.e., TCA converting to DCA). With any method developed, it is necessary to ascertain whether or not anything is happening to the analyte(s) in order to have confidence that a method is accurately measuring the analyte(s) it is designed to measure. In this section, several potential sources of species interconversion and analyte loss will be discussed.

Derivitization. The majority of methods for GC analysis of HAAs require a derivitization step. In this process, a HAA is commonly derivitized to its methyl ester in order to make the hydrophilic HAAs more volatile for GC analysis. A mixture of sulfuric acid, methanol, and water is one of the most common derivitizing agents. One study, however, found that using sulfuric acid in a derivitizing reagent for HAA analysis results in the conversion of TCA to DCA (Ketcha *et al*. 1996). The authors found that freezing blood samples prior to derivitization circumvents this problem by hemolyzing red blood cells and facilitating the oxidation of Fe(II) to Fe(III). Lead acetate has also been added to samples in order to prevent the conversion of TCA to DCA by quenching enzymatic reactions (Narayanan *et al*. 1999). Ketcha *et al*. (1996), however, determined that the addition of lead acetate to samples derivitized with sulfuric acid resulted in 80% conversion of TCA to DCA. Conversion of TCA to DCA remains one of the main concerns with HAA analysis today.

Acidification. Several methods acidify HAAs in order to convert them to their acidic form. The pK_as of TCA and DCA are approximately 0.5 and 1.5, respectively. This means that at pH 0.5, 50% of TCA is protonated and 50% is deprotonated. Most methods in which acidification is performed acidify samples containing HAAs to a pH of 0.5 or a more vague <0.5. It may be possible to convert nearly all of the HAAs to their acidic form by performing LLE several times, hopefully leaving the deprotonated HAAs in the water layer to be acidified upon removal of the protonated HAAs in the organic layer (due to equilibrium processes). Much higher levels of TCA have been shown to form in the presence of chlorine ions (Dalvi *et* al. 2000). This indicates that using HCl for sample acidification may convert DCA to TCA. Shorney and Randtke (1994) similarly report increased speciation shifts when HCl (as compared to sulfuric acid) is used with MeOH in a method for the analysis of HAAs. The most important point to make is simply that individuals performing acidification in methods for the analysis of HAAs should be aware of the pK_as of the HAAs, percentages of protonated and deprotonated species, and potential chemical reactions prior to beginning method development.

Enzyme activity. When analyzing biological samples for TCE and its metabolites, it is important to consider the possibility of enzymes converting one chemical entity to another *after* samples are collected. TCA, as a long-lived metabolite (Merdink *et al*. 1998), may be metabolized to DCA after samples have been collected. Another possibility is that DCA may be further metabolized after sample collection. There is difficulty in monitoring DCA *in vivo* in time-course studies of TCE metabolism, leading to the theory that DCA is a short-lived metabolite (Merdink *et al*. 1998). A method to stop metabolism at the time of collecting samples would be ideal for preserving the chemical species found in samples. Some possibilities for

stopping metabolism at sample collection time include: freezing with liquid nitrogen, heating the sample to denature enzymes, and protein precipitating samples as soon as possible after sample collection. Studies need to be performed with any method to evaluate the extent to which metabolism of analytes occurs after sample collection.

Solvent selection. Another area in which care needs to be taken is in solvent selection. TCE is commonly used as a degreaser and general-purpose solvent. Several companies use TCE to clean the equipment in which solvents are made and processed. As a result, many solvents are contaminated with TCE. One study has shown that MTBE is contaminated with very high levels of TCE (Brown *et al*. 2003b). The level of contamination varied from manufacturer to manufacturer and from batch to batch. It is possible to distill contaminated solvents in order to remove TCE contamination. However, finding a reliable manufacturer with low levels of TCE contamination and testing solvent blanks for TCE are the best ways to minimize solvent contamination.

VALIDATION

Overview. Results obtained from experiments are less meaningful if they contain too much error. Validating a method allows one to know the amount of error and therefore the confidence associated with the method and results. The USFDA has a set of guidelines that pharmaceutical industries must follow in validating any method developed for the analysis of pharmaceutical agents. This set of guidelines is strict, necessarily, because lives are literally on the line. While validations for environmental applications do not have to be so strict, the USFDA guidelines are

quite helpful for determining criteria for validating a method. Several parameters associated with developing an analytical method are listed in the USFDA guidelines and include: precision, accuracy, recovery, autosampler stability, and freeze/thaw stability. Each of these aspects of method validation will be discussed in this section.

Precision. Precision is the ability to obtain the same results several times. A precise method will give results in which all replicate samples have close to the same response (small standard deviation), but the calculated concentration may or may not be correct (accurate). Some methods report precision as a standard deviation of several equal concentration samples. This method of measuring precision does not give the quantitative precision of a method. The USFDA (2001) guidelines suggest measuring the precision as the percent coefficient of variation (%CV) for 5 samples each (n = 5) at several concentrations in the range of the calibration curve for the method. The %CV is measured as standard deviation/mean times 100, or (s/x)*100. This procedure should be performed a total of three times on three different days. Each set of 5 samples (n = 5) measures intraday precision and the total of 15 samples (3 sets of n = 5) measures interday precision of the method. The guidelines suggest measuring the precision at 5 concentrations: the LLOQ, 3 times the LLOQ, the ULOQ, 80% of the ULOQ, and some concentration near the middle of the calibration curve range. The criteria suggested for a concentration to pass precision validation is that the %CV is less than 20% for the LLOQ and less than 15% for all other validation points.

Accuracy. Accuracy is the ability to obtain correct results with a given method. While the average calculated concentrations of replicate samples may be accurate, the samples can still

have unacceptable precision. Many studies exist in which the accuracy is determined by recovery. This, however, does not give a quantitative representation of the error associated with the accuracy of a method. Several papers report accuracy values in terms of percent bias. The method by which percent bias is calculated can make results look more accurate than they are in reality. The USFDA (2001) guidelines for method validation suggest using percent error as a measure of accuracy. Percent error is calculated as the absolute value of the actual concentration minus the observed concentration divided by the actual concentration times 100, or, ((ABS(A-O))/A)*100. As with precision, the USFDA recommends a n = 5 at 5 validation points for intra-day validation and a n = 15 at the same validation points for inter-day validation. The criteria for passing validation are a %error less than 20% for the LLOQ and less than 15% for all other validation points.

Recovery. Recovery is the ability to extract an analyte from a matrix for a given method. There are two types of recovery—relative recovery and absolute recovery. Absolute recovery calculates the effect the matrix has on recovery, while relative recovery quantitates the efficiency of the extraction. Many papers report recovery, but do not contain information on how recovery studies were performed or what type of recovery was calculated. In addition, it is preferable to report the standard deviation or %RSD associated with the recovery as a measure of extraction reproducibility. Absolute recovery is calculated as the response for a sample in a matrix divided by the response for an equal concentration solvent standard times 100. Relative recovery is the ratio of the response of a sample in a matrix spiked before extraction, divided by the response of an equal concentration sample in a matrix spiked after extraction times 100. Calculating and

reporting both types of recovery provides more useful information about sample preparation and extraction than calculating only one of the two types of recovery.

Autosampler stability. Autosampler stability studies should also be performed on analyte(s) to ensure that the concentration of sample remains the same (or almost the same) from the time it is placed into the autosampler to the time at which it is actually analyzed (USFDA 2001). Several samples of the same concentration in a given matrix should be prepared and analyzed as they normally are. When the samples are to the point where they are ready to be placed into autosampler vials, the samples are pooled and mixed well prior to pipetting them into autosampler vials. This ensures that all samples are as close together in concentration as possible, minimizing the possibility that error in sample preparation leads to erroneous results. The samples should be injected at designated time intervals over a total length of time greater than or equal to the maximum time anticipated for real samples to run. If the response generally remains constant, then the samples are stable in the autosampler.

Freeze/Thaw Stability. Freeze/thaw stability of spiked tissue samples should be checked. This allows one to know the stability of samples in the event samples are stored in a freezer prior to analysis or need to be re-analyzed at a later time. The USFDA (2001) recommends spiking three samples in each matrix with a high concentration solution and three samples in each matrix with a low concentration solution. The samples are frozen for 12-24 hours and then allowed to thaw. One of the three samples for each group is analyzed (representing one freeze-thaw cycle) and the other two are frozen again. Data is obtained for a total of three freeze-thaw cycles.

Ion Suppression. Ion suppression studies are important when mass spectrometry is the means of detection. Ions and molecules present in biological matrices can cause suppression in the mass spectrometer source. Salts are a major source of ion suppression and can be found in most every biological matrix and in the mobile phase (buffers). Electrospray ionization, the most common type of ionization used with LC-MS analysis, is particularly susceptible to ion suppression. Studies should be performed to determine if and when (what retention time) ion suppression occurs. This knowledge allows for the opportunity to adjust chromatography so that the compound does not elute in the ion suppression region(s). A widely accepted method for performing ion suppression studies requires an aqueous solution of a selected concentration of analyte to be added post-column to the mobile phase, so that there is a constant infusion of analyte (King *et al*. 2000). A blank sample, which is in the matrix to be tested and has undergone the same sample preparation as a real sample in the given matrix, is then injected. The resulting chromatogram will be flat where no suppression occurs and have dips where the blank (matrix) causes ion suppression. If the elution time of the analyte in a normal run is not in the ion suppression region (where the dips are), then the chromatography is suitable for obtaining maximum analyte response. Matuszewski *et al*. 2003 further provide a method of quantitating the amount of ion suppression by determining the matrix effect. The authors determine matrix effect by dividing the response of a sample in a given matrix spiked after extraction by the response of an equal concentration solvent standard and then multiplying by 100. Values greater than 100% represent ion enhancement, while values less than 100% represent ion suppression. Subtracting the matrix effect from 100 should give the percent ion suppression or enhancement (negative values indicate ion enhancement).

LOD and LLOQ. The limits of detection and limits of quantitaion should be determined for the compound(s) in a study. The LLOQ rather than an LOD or MDL is more useful information, because the LLOQ is the lowest concentration that could actually be quantitated, as opposed to the lowest concentration that the instrument could detect (LOD) or a calculated MDL. In addition, the method by which the LOD and LLOQ are determined should be stated. Many studies determine a MDL, or minimum detection limit. Several methods are used to calculate the MDL, such as 3.14 times the standard deviation of replicate samples (typically about 5 times the concentration of the MDL). Still another reported method for determining the LOD or LOQ is simply 5 or 10 times the standard deviation of the blank. These means of calculating the LOD or LLOQ are subjective and are not based on signals observed from samples with analyte concentrations equal to the LLOQ. Having an accurate method for determining the LLOQ is particularly important, because it is critical to have confidence in the lowest concentration of an analyte(s) that can be accurately measured by a given method. The USFDA (2001) recommends determining the LLOQ as the lowest concentration sample at which one can obtain less than or equal to 20% precision (%CV) and accuracy (%error). Even if these standard guidelines are not used, it is important for any method to provide minimum acceptance criteria.

FUTURE DIRECTIONS

Overview. Various techniques involving separation, detection, sample preparation, and validation of TCE and its metabolites have been discussed. Over time, new analytical methodologies are discovered and are available for use by the scientific community as a whole. As a result, trends emerge in which certain analytical techniques become more prevalent within a

given field. This section discusses analytical techniques that are either appearing to be used more frequently or have good potential for use in the analysis of TCE and its metabolites.

SPME. As mentioned previously, SPME is a relatively new extraction technique that is particularly useful for volatile analytes. Over the past 15 years, the number of studies using SPME (mostly with GC) has continued to grow. As would be expected, the number of methods for the analysis of TCE and HAAs by SPME has also increased. The prevalence of reliable SPME autosamplers has helped SPME growth. SPME autosamplers have the ability to agitate and heat samples and to place the SPME fiber in the sample headspace and injection port for programmed lengths of time. SPME is a technique in which analytes are preconcentrated prior to injection. As such, SPME is often used in place of older sample preconcentration techniques such as LLE and purge and trap. Straightforward sample preparation and minimal solvent use make SPME an attractive alternative to LLE. With SPME, the sample is simply placed into a vial and capped prior to sample analysis. In contrast, LLE requires adding organic solvents to samples, often centrifuging them, and then transferring them to an autosampler vial. Purge and trap analysis has several problems associated with it such as carryover, low throughput, and a significant manpower commitment. In addition, SPME has an advantage over traditional headspace GC analysis in that SPME preconcentrates the sample, whereas headspace analysis does not. With headspace analysis, a volume of air from the headspace is drawn into the needle and whatever concentration of TCE is present in the headspace is also drawn into the needle. However, with SPME, TCE has a chemical affinity for the fiber and is thus preconcentrated onto the fiber prior to injection into the GC. As a result of all of the advantages that SPME offers, it

is likely that the number of methods using SPME as an extraction technique will increase for TCE and to a lesser extent for its (derivitized) metabolites.

HILIC. HILIC, like SPME, has been gaining popularity. As was mentioned in the LC section, traditional HPLC columns generally do not retain small polar compounds such as DCA and TCA well enough for sample analysis. Many compounds such as HAAs and small polar drugs are good candidates for HILIC and HILIC-ion exchange chromatography. Because the pK_as of DCA (~1.5) and TCA (~0.5) are low, the HAAs are almost always ionized and carry a negative charge. DCA and TCA should, then, interact with positively charged sites on amino columns. Since DCA and TCA are already in the (ionic) form needed for HILIC/HILIC-ion exchange analysis, there is no need for derivitization. This avoids potential issues with TCA being converted to DCA upon derivitization. In addition, the usefulness of both HILIC and HILIC-ion exchange with several small polar compounds, including DCA, has been illustrated (Olsen 2001, Brown *et al*. 2002, Grumbach *et al*. 2003, Schlichtherle-Cerny *et al*. 2003, Strege *et al*. 2000, Naidong 2003, Dixon *et al*. 2004). Due to the suitability of HILIC for HAA analysis, more studies with DCA and TCA using HILIC and/or HILIC-ion exchange chromatography are likely to be published.

FAIMS. High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) is another relatively new technique that shows potential for use in HAA analysis. FAIMS methods published for the analysis of HAAs report very low LODs for HAA9 (10 pg/mL for DCA and 36 pg/mL for TCA) in water samples. In addition, this technique separates HAAs much more quickly than HPLC when flow injection is performed (no separation technique used). As with

HPLC, no derivitization of HAAs is needed, because the ionized compound is in the form needed for FAIMS analysis. One drawback to FAIMS analysis, however, is that a separation technique is needed for biological samples. HPLC would be most commonly interfaced to FAIMS-MS for bioanalytical applications. FAIMS sources also use ESI at the LC-MS interface. Therefore, FAIMS faces the same chromatographic challenges and ion suppression as other LC-MS methods.

HPLC. The majority of methods for the analysis of TCE and its metabolites have up to this point been GC-MS methods. HPLC is likely to be used more in the future for the analysis of HAAs, but not for TCE. TCE is very lipophilic and volatile and, therefore, not compatible with HPLC systems. DCA and TCA, in contrast, are naturally compatible with HPLC analysis (do not need derivitization prior to analysis). In addition, more information is now available about columns and mobile phases that are suitable for HAA analysis. LC-MS methods for the analysis of HAAs will likely be found more often than LC-UV methods, because LC-MS, like GC, is much more sensitive than LC-UV for the analysis of TCE and its metabolites. The growing awareness of HILIC, HILIC-ion exhange, and the advantages of not derivitizing samples are likely to make HPLC methods for DCA and TCA more common.

GC. GC methods for the analysis of TCE and its metabolites will most likely still be prevalent. GC is the separation technique of choice for TCE. In addition, not everyone has a LC-MS available for HAA analysis. Large databases of samples run by GC-MS or GC-ECD exist. Therefore, it is likely that researchers will perform sample analysis by GC in order to both add to this database and to compare data they obtain to previously obtained GC data. In addition, the

advent of linear ion trap mass spectrometers will likely lead to a greater number of GC-MS applications using these instruments.

Validation. The importance of method validation cannot be stressed enough. Results obtained from a study may be meaningless if the method is not accurate and reproducible. Erroneous results can be obtained from species interconversion, irreproducibility of the method, degradation of an analyte over time, or for any number of other reasons. The error associated with the results may not be noticed if method validation is not performed. This has been shown to be particularly true for methods designed for the analysis of TCE and its metabolites (particularly for DCA and TCA). Validating a method, in contrast, instills confidence in both the method itself and results obtained in studies using the method. The value of having confidence in the method and results is well worth the time and effort of performing method validation. In addition, many journals are receiving an increasing number of articles for potential publication. Method validation increases the strength of a method by providing objective criteria by which to compare various methods. However, most toxicology journals, in which many studies of TCE and its metabolites are found, do not place much emphasis on analytical methods. Hopefully for all of the reasons mentioned above, method validation will become more common for future toxicological studies.

CONCLUSIONS

Issues associated with the potential carcinogenicity of TCE and its metabolites have been debated for the past several decades. In order to obtain accurate information relevant to the risk

assessment of TCE, DCA, and TCA, it is necessary to have good analytical methods with which to perform laboratory animal and human studies. Many methods exist for such analyses and have produced valuable information. Several of these methods can be further improved in order to obtain even more accurate results. In particular, taking care to preserve speciation of analytes and to perform method validation can substantially increase the confidence in a method. Several new types of technology have been introduced which have the potential to offer alternative methodologies and/or additional advantages in the analysis of TCE, DCA, and TCA. SPME, FAIMS, and HILIC should, in particular, provide for more accurate analysis of DCA and TCA, which have been very difficult to analyze in the past. Subsequent chapters in this dissertation further demonstrate the usefulness of SPME and HILIC in the analysis of TCE and DCA. Chapter 2 illustrates the usefulness of SPME in GC/MS analysis of TCE. Chapters 3 and 4 provide validated methods for DCA in drinking water and blood and tissue samples using HILIC-LC/MS. Use of newer technologies such as SPME and HILIC, as demonstrated in Chapters 2-4, should in turn allow for more accurate data to be obtained and utilized in future risk assessments of TCE and its metabolites.

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Table 1.1 Acronyms used in this review paper

ACN	Acetonitrile
ATSDR	Agency for Toxic Substances and Disease Registry
BDCA	Bromodichloroacetic acid
BF_3-MeOH	Boron trifluoride-methanol complex, a derivitizing reagent
C2	Two-carbon long alkyl chain
C8	Eight-carbon long alkyl chain
C18	Octadecyl, eighteen-carbon long alkyl chain
CAR	Carboxen
CE	Capillary electrophoresis
CE-UV	Capillary electrophoresis with ultraviolet detection
CE-MS	Capillary electrophoresis interfaced with a mass spectrometer
CH	Chloral hydrate
CTAB	Hexadecyltrimethylammonium bromide
CTAC	Cetyltrimethylammonium chloride
CV	Coefficient of variation
CZE	Capillary zone electrophoresis
DBA	Dibromoacetic acid
DCA	Dichloroacetic acid
DCE	Dichloroethylene
DVB	Divinylbenzene
ECD	Electron capture detection
EOF	Electroosmotic flow
ECNCI	Electron capture negative ion chemical ionization
ESI	Electrospray ionization
ESI-MS	Use of electrospray ionization in the source of a mass spectrometer
ESI-FAIMS-MS	High-field asymmetric waveform ion mobility spectrometry used in conjunction with a mass spectrometer equipped with electrospray ionization
FAIMS	High-field asymmetric waveform ion mobility spectrometry
FAIMS-MS	High-field asymmetric waveform ion mobility spectrometry used in conjunction with a mass spectrometer
FID	Flame ionization detection
GC	Gas chromatography
GC-ECD	Gas chromatography with electron capture detection
GC-FID	Gas chromatography with flame ionization detection
GC-MIMS	Gas chromatography coupled to a mass spectrometer with a membrane inlet
GC-MS	Gas chromatography interfaced with mass spectrometry
HAA	Haloacetic acid
HAA5	Five haloacetic acids regulated by the EPA: monochloroaceticacid, dichloroacetic acid, trichloroacetic acid, bromoacetic acid, dibromoacetic acid
HAA9	Nine haloacetic acids: monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid,

	dibromoacetic acid, tribromoacetic acid, bromochloroacetic acid, dibromochloroacetic acid, dichlorobromoacetic acid
HCl	Hydrochloric acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography with ultraviolet detection
IC	Ion chromatography
IPA	Isopropanol
LC	Liquid chromatography (high performance)
LC-UV	Liquid chromatography (high performance) with ultraviolet detection
LC-MS	Liquid chromatography (high performance) interfaced with mass spectrometry
LC-MS-MS	Liquid chromatography (high performance) interfaced with tandem mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LOQ	Limit of quantitation
MBA	Monobromoacetic acid
MCA	Monochloroacetic acid
MCL	Maximum contaminant level
MCLG	Maximum contaminant level goal
MDL	Minimum detection limit
MQL	Minimum quantification limit
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MTBE	Methyl tert-butyl ether
NDC	2,6-napthalenedicarboxylic acid dipotassium
NOM	Natural organic matter
PCE	Perchloroethylene, also known as tetrachloroethylene
PDMS	Polydimethylsiloxane
PP	Protein precipitation
Q1	First quadrupole on a triple quadrupole mass spectrometer
Q3	Third quadrupole on a triple quadrupole mass spectrometer
QC	Quality control
RP	Reversed phase
RSD	Relative standard deviation
SAX	Strong anion exchange
SCX	Strong cation exchange
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
S/N	Signal-to-noise ratio
SPE	Solid phase extraction

SPME	Solid phase microextraction
SPME-GC-ECD	Solid phase microextraction used in conjunction with a gas chromatograph equipped with an electron capture detector
TCA	Trichloroacetic acid
TCA-2-^{13}C	Radioactively-labeled trichloroacetic acid, with a carbon-13 on the second carbon
TCE	Trichloroethylene
TCEOH	Trichloroethanol
TOF-MS	Time-of-flight mass spectrometer
ULOQ	Upper limit of quantitation
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
UV	Ultraviolet
VOC	Volatile organic compound
WAX	Weak anion exchange
WCX	Weak cation exchange

Table 1.2 Analytical Methods for the Analysis of TCE and its Metabolites

Author(s)	Analyte	Matrix	Separation Type	Detector	LOD or LOQ
Munch and Hautman	TCE	Water	GC	ECD	0.002 ng/mL (MDL)
Munch (ed.)	TCE	Water	GC	MS	0.02 ng/mL (MDL)
Eichelberger *et al.*	TCE	Water	GC	MS	5 ng/mL (MDL)
Brown *et al.* 2003a	TCE	Water	GC	MS	5 ng/mL (LLOQ)
Bocchini *et al.*	TCE	Water	GC	MS	N/A
Xu *et al.*	TCE	Water	GC	FID	5 ng/mL (LOD)
Domino *et al*.	DCA, TCA	Water	GC	ECD	0.02 ng/mL (MDL, DCA) 0.019 ng/mL (MDL, TCA)
Xie	DCA, TCA	Water	GC	MS	0.07 ng/mL (MDL, DCA) 0.15 ng/mL (MDL, TCA)
Berg *et al.*	DCA, TCA	Water	GC	MS	1 ng/mL (LOD, DCA) 4 ng/mL (LOD, TCA)
Sarríon *et al.*1999	DCA, TCA	Water	GC	MS	0.04 ng/mL (LOD, DCA) 0.01 ng/mL (LOD, TCA)
Sarríon *et al.* 2000	DCA, TCA	Water	GC	MS	0.07 ng/mL (LOD, DCA) 0.02 ng/mL (LOD, TCA)
Wong *et al.*	DCA, TCA	Water	GC	MS	N/A
Dalvi *et al.*	DCA, TCA	Water	GC	ECD	0.5 ng/mL (LOD, DCA) 0.1 ng/mL (LOD, TCA)

Benanou *et al.*	DCA, TCA	Water	GC	ECD	0.15 ng/mL (LOD, DCA) 0.07 ng/mL (LOD, TCA)
Merdink *et al.*	TCE, CH, DCA, TCA	Blood	GC	ECD	65.4 ng/mL (LLOQ, TCA) 245 ng/mL (LLOQ, DCA) Not reported (TCE and CH)
Song and Ho	TCA, TCEOH	Urine	GC	MS	2.3 ng/mL (LLOQ, TCA) 1.7 ng/mL (LLOQ, TCEOH)
Forkert *et al.*	TCE, TCEOH, DCA, TCA	Seminal fluid	GC	ECD	N/A
Ashley *et al*. 1992	TCE	Blood	GC	MS	0.005 ng/mL (LOD)
Ashley *et al*. 1994	TCE	Blood	GC	MS	0.01 ng/mL (LOD)
Brown *et al.* 2003b	TCE	Lung, Liver, Kidney	GC	MS	5 ng/mL (LLOQ)
Muralidhara and Bruckner	TCE, TCA, DCA, TCEOH	Lung, Liver, Kidney, Blood	GC	ECD	5 ng/mL (LOD, TCE) 5 ng/mL (LOD, TCA) 10 ng/mL (LOD, DCA) 5 ng/mL (LOD, TCEOH)
Dehon *et al.*	TCE, TCA,	Blood, Urine, Multiple tissues	GC	ECD	N/A
Yan *et al.* 1997	DCA, MCA	Plasma	GC	MS	74.8 ng/mL (LLOQ, DCA) 204.1 ng/mL (LLOQ, MCA)
Yan *et al.* 1999	CH, MCA, DCA, TCA, TCEOH	Plasma	GC	MS	1295.1 ng/mL (LOQ, CH) 17.0 ng/mL (LOQ, MCA) 15.5 ng/mL (LOQ, DCA) 1248.3 ng/mL (LOQ, TCA) 934.2 ng/mL (LOQ, TCE)

Kim *et al.*1999	TCA, DCA	Urine	GC	ECD	N/A
Ketcha *et al.*	DCA, TCA	Blood	GC	ECD	N/A
Jia *et al.*	TCA, DCA	Plasma, Urine	GC	MS	0.05 ng/mL (LOD, DCA) 1 ng/mL (LOD, TCA)
Wu *et al*.	TCA, DCA	Urine, Blood, Water	GC	ECD	0.6 ng/mL (MDL, DCA) 0.6 ng/mL (MDL, TCA)
Kuklenyik *et al.*	TCA	Urine	HPLC	MS-MS	1.7 ng/mL (LOQ)
Carrero and Rusling	DCA, TCA	Water	HPLC	Electro-chemical	4000 ng/mL (LOD, DCA) 120 ng/mL (LOD, TCA)
Loos and Barcelo	DCA, TCA	Water	HPLC	MS	1.1 ng/mL (LOQ, DCA) 1.3 ng/mL (LOQ, TCA)
Takino *et al*.	DCA, TCA	Water	HPLC	MS	1.07 ng/mL (MQL, DCA) 1.15 ng/mL (MQL, DCA)
Narayanan *et al.*	DCA	Plasma, Urine	HPLC	Conductivity	50 ng/mL (LOD)
Hashimoto and Otsuki	DCA, TCA	Water	HPLC	MS	0.003 ng/mL (LOD, DCA) 0.070 ng/mL (LOD, TCA)
Dixon *et al*.	DCA	Water	HPLC	MS-MS	5 ng/mL (LLOQ, DCA)
Ahrer and Buchberger	DCA, TCA	Water	CE	MS	0.3 ng/mL (LOD, DCA) 0.5 ng/mL (LOD, TCA)
Kim *et al.* 2001	DCA	Water	CE	UV	N/A
Martinez *et al.* 1998a	DCA, TCA	Water	CE	indirect UV	2 ng/mL (LOD, DCA) 2 ng/mL (LOD, TCA)
Martinez *et al.* 1998b	DCA, TCA	Water	CE	indirect UV	0.15 µg/mL (LOD, DCA) 0.50 µg/mL (LOD, TCA)

Martinez *et al.* 1999	DCA, TCA	Water	CE	indirect UV	N/A
Sarzanini *et al.*	DCA, TCA	Water	IC	UV	7 ng/mL (LLOQ, DCA) 10 ng/mL (LLOQ, TCA)
Qu and Mou	MCA, DCA	Water	IC	Conductivity	25 ng/mL (LOD, DCA)
Ko *et al.*	DCA, TCA	Water	IC	Conductivity	0.45 ng/mL (MDL, DCA) 1.5 ng/mL (MDL, TCA)
Ells *et al.*	DCA, TCA	Water	FAIMS	MS-MS	10 pg/mL (LOD, DCA) 36 pg/mL (LOD, TCA)
Gabryelski *et al.*	DCA, TCA	Water	FAIMS	MS	0.60 ng/mL (LOQ, TCA) 0.55 ng/mL (LOQ, DCA)
Brashear *et al.*	MCA, DCA, TCA	Blood	none	MS-MS	4 ng/mL (LOD, DCA) 4 ng/mL (LOD, TCA) 25 ng/mL (LOD, MCA)
Magnuson and Kelty	DCA, TCA	Water	none	MS-MS	0.32 ng/mL (MDL, DCA) 0.13 ng/mL (MDL, TCA)
Debré *et al.*	DCA, TCA	Water	none	MS	N/A

CHAPTER 2

OPTIMIZATION OF SPME FOR ANALYSIS OF TRICHLOROETHYLENE IN RAT BLOOD AND TISSUES BY SPME-GC/MS

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ABSTRACT

Trichloroethyene (TCE) is a small halogenated compound that has been used extensively as a metal degreaser, dry cleaning agent, and all-purpose solvent. As a result of its widespread use, TCE is commonly found in the environment. TCE has limited water solubility, therefore the compound is able to partition into drinking water. Human exposure to TCE in environmental media is of concern, because TCE has been found to be carcinogenic in laboratory animals. The method presented in this paper uses solid phase microextraction coupled with gas chromatography and mass spectrometry (SPME-GC/MS) to analyze TCE in rat blood, plasma, liver, kidney, and lung. SPME pre-concentrates the sample, minimizes the use of extraction solvents, and minimizes sample preparation time. The limit of detection for this method is 1 ng/mL. For all biological speicmens, placing the fiber in the sample headspace for 5 minutes for extraction of TCE gave maximal detector response. For blood and liver samples, heating samples prior to inserting the fiber improved the sensitivity. It is believed that sample heating overcomes TCE binding to heme-containing proteins in these matrices. The length of time that the sample was heated had no effect on TCE response. Lung and kidney samples, due to the low amounts of heme-containing proteins, yielded better responses when not heated at all.

INTRODUCTION

Trichloroethylene (TCE) is a volatile compound that has been used extensively as a dry cleaning agent and metal degreaser. As a result of its widespread use, volatility, and limited water solubility, TCE is found extensively in the environment. In fact, TCE has been found at 50% of

the U.S. Environmental Protection Agency's (USEPA) Superfund sites.(1) TCE has been a chemical of concern for decades, because studies have shown that the compound is a liver carcinogen in mice and a kidney carcinogen in rats.(2,3) In most areas in the United States, TCE is present below the USEPA regulatory limit of 5 ng/mL in drinking water. There are numerous exceptions. Levels of up to 440 ng/mL TCE have been found in wells, with Los Angeles County having a high occurrence of contaminated wells.(1) Exposure to TCE from drinking water occurs by three routes: 1) ingestion of drinking water, 2) dermal absorption (mostly while showering) and 3) inhalation due to volatilization of TCE while taking a hot shower or cooking.(1,4) TCE has been found in air and water in increasing amounts in the following types of environments, respectively: forest, rural, city, and commercial.(1) As would be expected, individuals exposed to TCE occupationally and those living near hazardous waste sites containing with the chemical experience the highest exposures to TCE. All 8 breast milk samples from women living in industrialized areas tested positive for TCE.(1) It has been estimated by the Centers for Disease Control (CDC) and others that 10-13% of the general population have detectable levels of TCE in their blood and urine.(1,5,6) The most common adverse effect associated with acute TCE inhalation is central nervous system depression. Symptoms can range from dizziness and headache to disorientation and unconsciousness.(2) At very high level exposures, cardiac arrhythmias can also occur.(2) Chronic, high level oral and inhalation exposures to TCE have been shown to cause liver cancer in mice and kidney cancer in rats.(2,3,7) Increased incidences of renal cell carcinoma have been reported in workers exposed occupationally for years to high vapor levels of TCE.(8,9,10) As a result, TCE has been listed as "reasonably anticipated to be a human carcinogen" in the National Toxicology Program's (NTP) Tenth Report on Carcinogens and as a "probable human carcinogen" by the International Agency for Research on Cancer

(IARC).(11,12) The USEPA is currently in the process of revising their cancer risk assessment of TCE. A key question is whether environmentally-encountered levels of TCE pose a cancer risk. In order to better evaluate the possible role of trace amounts of TCE in carcinogenesis, it is very important to have reliable analytical methods for the quantitation of minute concentrations of TCE in biological matrices.

Solid phase microextraction (SPME) is a relatively new technique introduced by Janus Pawliszyn in the late 1980s. With this method, a SPME fiber is either introduced into the sample headspace (gaseous region above the liquid sample in a vial) or immersed directly into the sample.(13) SPME headspace sampling is usually used when the analyte is in a biological matrix, because this prolongs the life of the fiber. Direct immersion of the SPME fiber in a biological sample results in binding of molecules such as proteins to the fiber, decreasing its lifetime. SPME is available for use with GC or LC applications. GC applications with SPME are the most common, because the fiber can concentrate volatile analytes (which are typically analyzed by GC). SPME fibers have a chemical coating on their surface. The chemical coating should be selected such that the analyte has a stronger affinity for the coating than the surrounding medium. Upon introduction, the analyte adsorbs onto the fiber. It is then thermally desorbed from the fiber in the GC injection port.

SPME has several advantages over classical GC techniques. In traditional headspace GC, a certain volume of air is simply pulled into the autosampler needle. Therefore, sensitivity is ultimately limited by the concentration of the analyte in the headspace. However, SPME can pre-concentrate the sample due to the high affinity of the analyte for the fiber coating. Additionally,

fewer organic solvents are used, as the solvents commonly used for extracting volatile analytes from biological samples are not needed. With SPME, sample handling is minimized. Once a biological sample is taken, it is simply placed into an autosampler vial and capped until analysis. This limited sample preparation also helps to minimize loss of volatile analytes and to reduce error in the analytical method, which typically increases with increasing number of sample manipulations. Having fewer sample preparation steps saves time, particularly when the sample preparation is not automated. Another important advantage with headspace SPME is that matrix effects should be reduced. Biological molecules, such as proteins, to which TCE may bind are located in the liquid portion of the sample, not in the sample headspace. Therefore, all TCE that is in the sample headspace should be available to adsorb onto the SPME fiber.

In order to better understand the carcinogenic potential of TCE, it is important to be able to perform animal experiments to elucidate the disposition of the chemical in the body. It is preferable to take serial blood samples from individual animals, in order to reduce variability and the number of animals that must be utilized. It is also necessary to minimize sample volumes when taking serial samples from small rodents. A limited number of methods have been published describing the use of SPME for TCE analysis.(14,15) One such method was used for analysis of TCE in a human forensic study.(14) Relatively large blood and tissue samples are available in such instances. The optimization of SPME for TCE, however, was not discussed in this paper. Another procedure involved the direct immersion of the SPME fiber in water samples.(15) This method works well for analysis of TCE in water, but direct immersion is not practical for most biological samples. Several methods also exist for the analysis of TCE in blood and water samples by purge and trap GC/MS.(6,16,17) These methods are quite sensitive,

but the sample volume used in these studies was 5 mL, which is not realistic for studies with small rodents. In addition, purge and trap analysis is problematic, in that it is tedious, time consuming, has low throughput, and introduces carryover problems.

Additional methods exist for GC analysis of TCE.(18,19,20) One uses headspace GC analysis with an electron capture detector (ECD).(20) Again, traditional headspace GC analysis is less sensitive than SPME, because the former technique does not involve sample pre-concentration. Another technique involves liquid-liquid extraction (LLE) with ether followed by GC/MS analysis of TCE.(18,19) It has been shown that many solvents used in LLE are contaminated with TCE.(19) This complicates efforts to quantify very low concentrations of TCE in biological specimens. These methods both involve the use of solvents and additional sample preparation steps. This results in increased cost, time, and potential error in the analysis. Therefore, the objectives of the current project were to optimize a SPME method for the analysis of TCE in rats that eliminates the need for solvents and reduces potential error while still allowing for sensitive detection of TCE and to compare this method to the LLE-GC/MS developed using the same instrumentation.

EXPERIMENTAL

Chemicals and Reagents

Analytical grade trichloroethylene (TCE) was purchased from Aldrich (Milwaukee, WI, USA). Perfluorokerosene, the calibrant for the mass spectrometer, was purchased from Sigma (St.

Louis, MO, USA). Deionized water used in experiments was generated from a Continental water deionization system (Natick, MA, USA). Ultra high purity (UHP) helium was purchased from National Welders (Charlotte, NC, USA) for use as the GC carrier gas. Solid phase microextraction fibers (100 μm polydimethylsiloxane coating) were purchased from Supelco (Bellefonte, PA).

SPME Method Development

Several parameters involved in SPME were varied to determine optimal conditions for TCE extraction and analysis. In order to determine the optimum temperature for sample desorption in the GC injection port, the polydimethylsiloxane (PDMS) fiber was placed into the GC injection port at temperatures of 50, 100, and 200^{o}C. Lengths of time for exposure of the SPME fiber to the sample headspace were 30 seconds and 1, 3, 5, 10, 15, and 25 minutes. Temperatures at which samples were heated prior to fiber insertion ranged from 25 to 95^{o}C in 10-degree increments. Lengths of time of heating samples before inserting the fiber were 0, 1, 3, and 5 minutes and 5-minute increments up to 60 minutes. Some samples were heated before placing the fiber into the headspace, and some samples were heated after placing the sample into the headspace in order to determine which technique yielded the highest detector response. Also, in order to determine relative responses, samples of equal concentrations of TCE were run in several different matrices and compared to one another. The matrices in which TCE was dissolved included: water and rat lung, liver, kidney, plasma, and blood.

Final GC Conditions

The GC/MS method used in this study was adapted from a LLE-GC/MS method for TCE that has been previously described.(18) An Agilent 5890 Series II GC (Palo Alto, CA) was used for this study. The GC column was a Phenomenex (Torrance, CA, USA) ZB-5 (30 m length x 0.25 mm i.d. x 25 μm film thickness) column. The column was run isothermally at 35^{o}C for all experiments. Helium was used as the carrier gas. The gas flow rate was 30 cm/min (pressure = 13.0 psi). The injection port temperature was maintained at 200^{o}C. The SPME fiber was inserted into the injection port and allowed to remain there for 10 seconds prior to starting the GC run.

Final Mass Spectrometer Conditions

The mass spectrometer used in this study was a Micromass Autospec Magnetic Sector Mass Spectrometer (Manchester, UK). The mass spectrometer was run in positive electron ionization (EI^{+}) mode with an electron energy of 70 eV and was tuned and calibrated daily. Perfluorokerosene (PFK) was used for instrument calibration. The mass spectrometer was operated in SIM (selected ion monitoring) mode at a resolution of 5000 (FWHM) in order to monitor the masses 129.9144 (TCE) and 130.99202 (PFK). The trap current used was 400 V.

Sample Preparation

An aqueous 100 μg/mL solution of TCE was made in water each day samples were run. One hundred μL of this solution was added to 900 μL of water, resulting in a concentration 10 μg/mL

TCE. This solution was used to spike all biological samples. All blood and tissue samples were obtained from male Sprague-Dawley rats. In an effort to remove residual blood from the lung, liver, and kidneys, cold saline was injected into the portal vein and exited via the severed inferior vena cava. Each sample consisted of 100 μL of blood, plasma, or homogenized lung, liver, or kidney placed into an autosampler vial. Plasma was obtained by separating whole blood at 16000 x g for 10 minutes. One hundred μL of 10 ug/mL aqueous TCE solution were added to each sample. This resulted in samples with concentrations of 5 μg/mL TCE. After spiking samples with TCE, each autosampler vial was immediately crimped tightly prior to SPME-GC/MS analysis.

RESULTS AND DISCUSSION

This SPME-GC/MS method for the analysis of TCE showed good agreement with the previously developed LLE-GC/MS method (Table 1). The TCE peak had approximately the same retention time (3.6 vs. 3.3 minutes), and peak heights were comparable between the two methods. The differences in retention times were due to leaving the SPME fiber in the injection port for 10 seconds before starting the run and trimming the column for maintenance purposes (different length column = different retention time). The same LOD (1 ng/mL) was obtained with both methods using the same starting volume of tissue homogenate (200 μL). LLE-GC/MS had recoveries from tissue samples that were 10-30% higher than the SPME-GC/MS method. One advantage of SPME-GC/MS analysis over LLE-GC/MS was the ability to measure TCE in blood samples. Liver, lung, and kidney samples were successfully analyzed for TCE by LLE-GC/MS. However, it was not possible to measure TCE in blood samples, perhaps due to interactions

between TCE and blood components. Other advantages of SPME-GC/MS over LLE-GC/MS were less sample preparation and no use of organic solvents. A representative chromatogram of TCE using SPME-GC/MS is shown in Figure 1. The optimum temperature for the injection port was found to be 200ºC, which is the temperature recommended by the manufacturer of the SPME fibers. When the injection port was maintained at 100ºC or less, a split peak was the result. This likely occurred because all TCE did not desorb from the SPME fiber at the same time. Measuring equal concentration samples in different matrices showed that liver, lung, kidney, and plasma sample responses were all approximately two-thirds that of water samples (Figure 2). This decrease is most likely due to binding to biological molecules. Blood samples had about half the response versus the other biological matrices. It has been shown that several VOCs bind to blood proteins.(21) The binding of TCE, to albumin and especially to hemoglobin, is likely the cause for lower TCE response from blood samples. Higher TCE responses were obtained when the sample was heated before inserting the fiber into the headspace (as opposed to having the fiber in the headspace while heating the sample). Having the SPME fiber in the headspace while heating the sample apparently warms the fiber and results in a decreased capacity to adsorb TCE.

For the biological matrices tested (lung, liver, kidney, blood), 5 minutes was found to be the optimum time to leave the fiber in the sample headspace (temperature and length of sample heating constant at 65ºC for 5 minutes prior to inserting fiber, Figure 3). This indicates that equilibrium between TCE in the liquid sample, headspace, and fiber coating is not established instantaneously. From 0-5 minutes the volatilized TCE is adsorbed onto the fiber. Additional TCE subsequently enters the headspace from the sample matrix in an effort to re-establish

equilibrium. However, over time the fiber warms due to sample heating, reducing the capacity of the fiber to retain TCE. The samples thus were heated before the fiber was inserted, in order to delay warming the fiber. This approach provided the greatest improvement for blood (25% increase in response) and liver (15% increase in response) samples compared to heating the sample with the fiber in the sample headspace.

Each biological matrix was tested over a range of temperatures (20–100°C, samples heated 5 minutes, fiber in headspace 1 minute) to determine the optimal temperature for SPME (Figure 4). Interestingly, each matrix had a different optimal temperature, demonstrating the need to test each matrix rather than assuming consistent behavior among them. Blood samples required the greatest heating, with 55°C providing the highest response. Liver samples required heating to 45°C. However, lung and kidney samples provided the highest response when not heated. The cause of the differences in sample heating parameters is believed to be related to protein binding. Proteins in blood such as hemoglobin and albumin have been shown to bind VOCs like TCE.(21) Support for the role of hemoglobin can be seen in Figure 2, where extraction from plasma is more efficient than from whole blood. The liver also requires significant heating. The liver processes hemoglobin from lysed erythrocytes and contains high levels of cytochrome P450s and other heme-containing proteins; therefore relatively high binding of TCE would be expected. The liver, of course, is the primary site of metabolism of TCE and many other xenobiotics. The levels of heme-containing proteins in the lung and kidney, however, are substantially lower in these organs than in the liver and blood. These organs have quite limited capacity to metabolize TCE due to their relatively low cytochrome P450 content.(22,23)

The initial increase in TCE concentration observed (at low temperatures) in the headspace for blood and liver samples is believed to represent TCE that was weakly bound to biological molecules. The secondary release observed at 75-85°C represents TCE released following denaturation of proteins in the samples. Evidence for thermal protein degradation was manifest by a color change in all samples (i.e. blood turned from red to brown). The instrument response for this secondary release was not as high as the TCE response at lower temperatures for all samples. This lower response is related to the observed loss of binding capacity for TCE on the fiber when the samples were heated. As expected, heating to temperatures above 90°C resulted in significantly decreased response for TCE.

Finally, the length of time that the sample was heated before fiber insertion was studied (for all samples temperature = 65°C, extraction time = 1 minute). In Figure 5, the heating time was varied from 0–30 minutes. There did not appear to be any effect on sample response over the time frame employed. The lack of sensitivity to sample heating may be related to rapid attainment of equilibrium between the sample and the headspace.

CONCLUSIONS

This SPME-GC/MS method was shown to work well for the analysis of TCE compared to the previously developed LLE-GC/MS method. The limits of detection, retention times, and peak heights, were comparable between the two methods. Ours is apparently the first SPME-GC/MS method optimized for the determination of TCE in biological specimens from laboratory animals. SPME eliminated the need for solvents and several sample preparation steps, resulting

in less potential for error and analyte loss with less waste generated compared to the traditional LLE-GC/MS analysis. SPME-GC/MS also has the capability to analyze TCE in blood samples. Sample pre-concentration by SPME made this method more sensitive than the headspace GC technique.(20) The limit of detection for SPME-GC/MS method was 1 ng/mL, the same as that of the LLE-GC/MS method and 50-fold lower than with headspace GC-ECD.(18,20)

ACKNOWLEDGEMENTS

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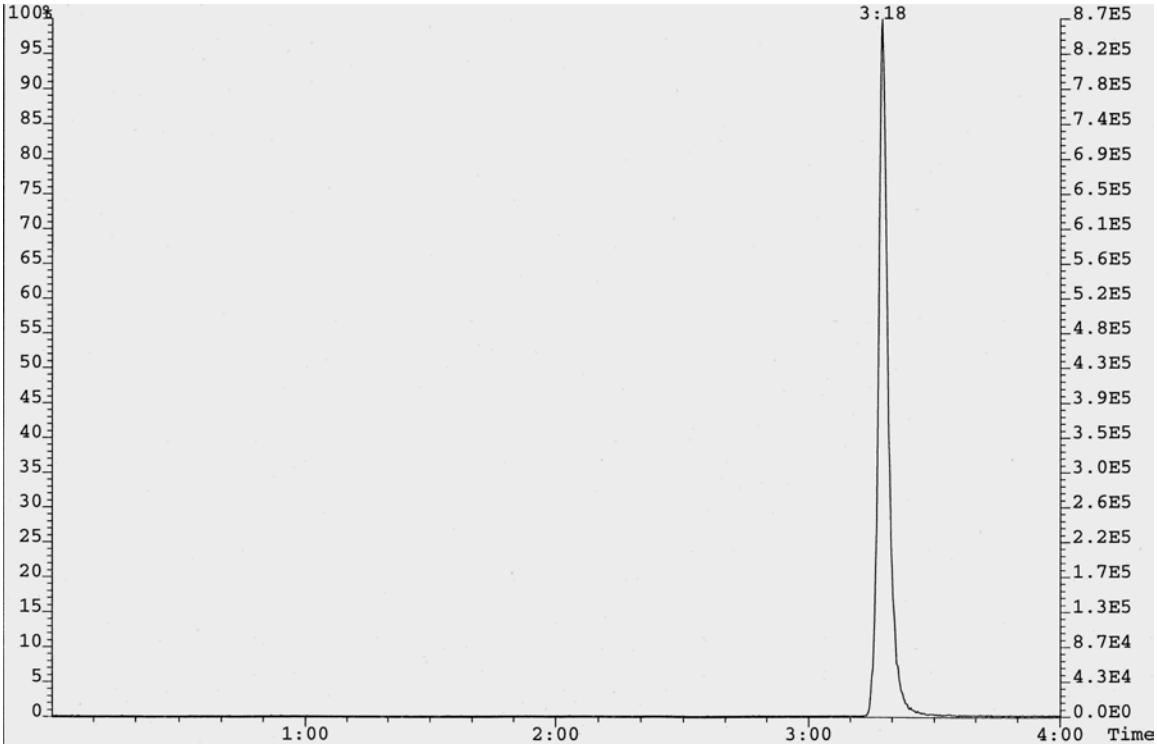

Figure 2.1 Representative Chromatogram of TCE in blood at 5 μg/mL.

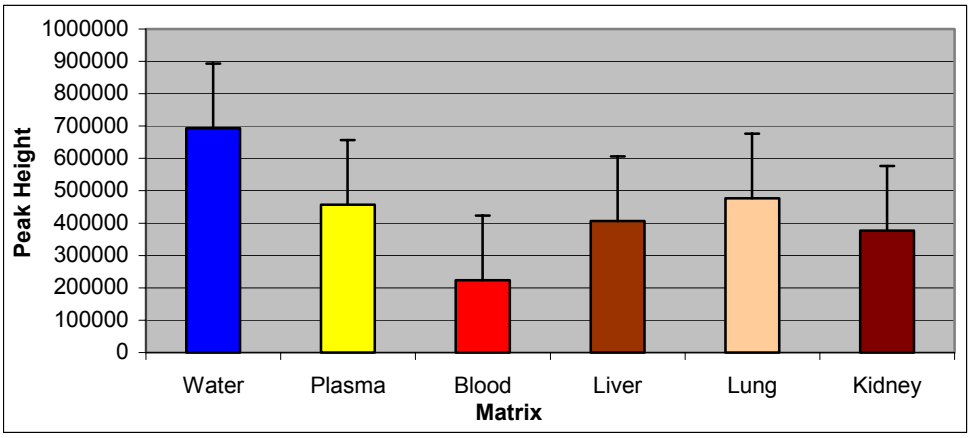

Figure 2.2 Comparison of TCE Response in Different Matrices.

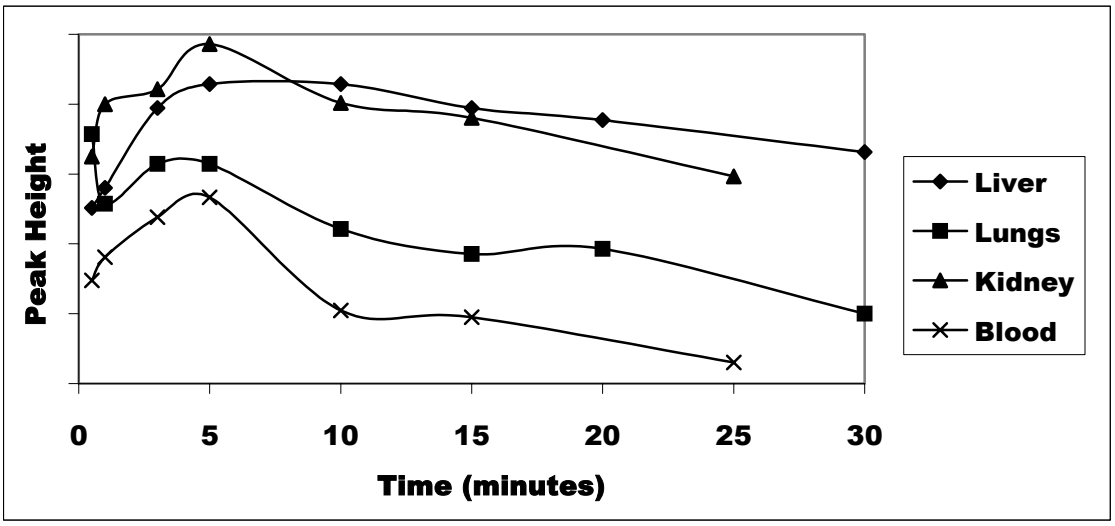

Figure 2.3 Optimization of Length of Time To Expose SPME Fiber to Sample Headspace at TCE Concentration of 5 μg/mL.

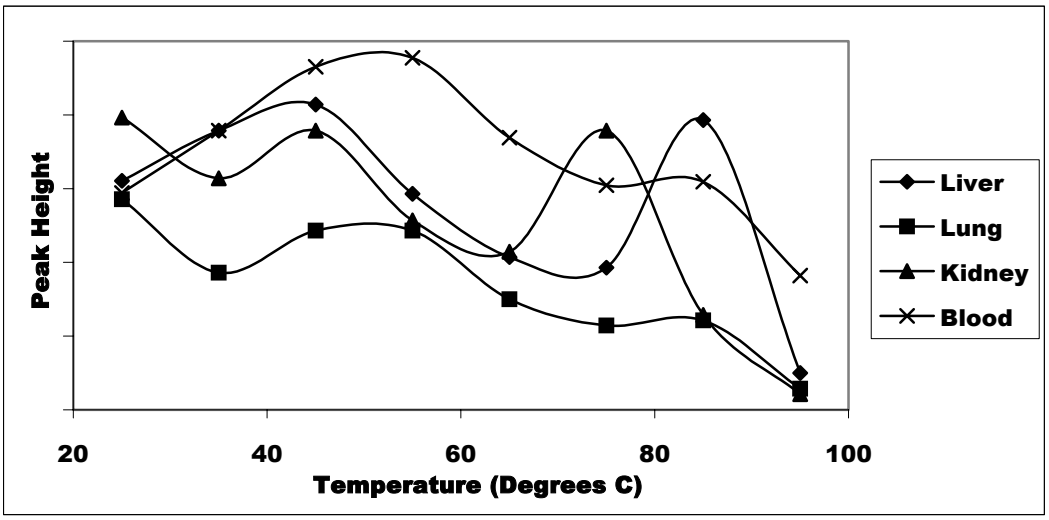

Figure 2.4 Optimization of Temperature at Which to Heat Sample at TCE Concentration of 5 μg/mL.

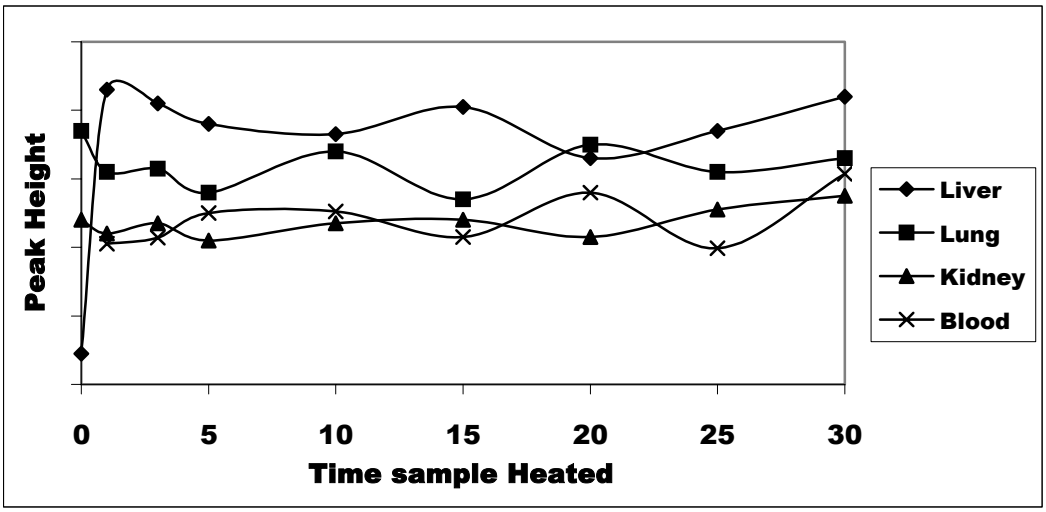

Figure 2.5 Optimization of Length of Time to Heat Samples at TCE Concentration of 5 μg/mL.

Table 2.1 Comparisons Between LLE-GC/MS and SPME-GC/MS Methods

PARAMETER	LLE-GC/MS	SPME-GC/MS
Sample size	200 uL	200 uL
Volume extraction solvent used	200 uL	No solvent used
Recovery		
Liver	87.2 ± 2.78	59.1 ± 11.6
Lung	79.2 ± 10.8	69.9 ± 7.0
Kidney	79.9 ± 14.2	54.7 ± 13.9
Blood	Not determined	32.4 ± 6.8
Plasma	Not determined	66.3 ± 13.2
Instrumental Method	GC/MS	GC/MS
Limit of detection	1 ng/mL	1 ng/mL
Retention time for TCE	3.6 min	3.3 min

CHAPTER 3

ANALYSIS OF DICHLOROACETIC ACID IN DRINKING WATER BY ION EXCHANGE HILIC-LC/MS/MS

Dixon, A.M., Delinsky, D.C., Bruckner, J.V., Fisher, J.W., and Bartlett, M.G. *Journal of Liquid Chromatography and Related Technologies*. 27: 2343-2355. Reprinted here with permission of publisher.

ABSTRACT

Dichloroacetic acid (DCA) is a small, polar compound that individuals are exposed to as a result of drinking water consumption. The occurrence of DCA in drinking water is of concern because DCA has been shown to cause cancer in laboratory animals. To date, no validated LC/MS/MS methods are available for quantitative analysis of DCA. In addition, most methods use a derivatizing reagent that can artificially inflate the levels of DCA. Presented in this paper is a validated LC/MS/MS method for the analysis of DCA in drinking water. An amino column was used with ion-exchange hydrophilic interaction chromatography (HILIC).

INTRODUCTION

Dichloroacetic acid (DCA) is a compound that has been recently receiving close attention from the United States Environmental Protection Agency (USEPA) due to its potential to adversely affect individuals who consume it in drinking water. There are two main sources of exposure to DCA. DCA is found in drinking water as a disinfection by-product of chlorination. A second source of exposure is from solvents that are metabolized to DCA. One such solvent is trichloroethylene (TCE), another common contaminant in drinking water. TCE has been used extensively as a metal degreaser, in a dry cleaning, and as a general-purpose solvent. As a result of its widespread use and the fact that it is soluble to some extent in water, TCE is a common contaminant of water supplies nationwide.(1) DCA is formed from metabolic conversion of TCE via the cytochrome p-450 pathway.(2,3)

Chlorination of drinking water containing tannins (natural organic matter) produces haloacetic acids (HAAs). The Maximum Contaminant Level (MCL) issued for total of 5 HAAs (HAA5) by the USEPA is 60 ng/mL in drinking water for the following acids: DCA, TCA (trichloroacetic acid), MCA (monochloroacetic acid), MBA (monobromoacetic acid) and DBA (dibromoacetic acid). DCA and TCA have been found to make up the majority of HAAs in drinking water.(4,5)

In the past it was believed that TCE itself was the cause of liver cancer in mice exposed to TCE in drinking water. More recently, it has been found that the metabolites of TCE are actually causing the observed carcinogenic effects.(6-9) Among the metabolites shown to cause cancer are DCA, TCA, and chloral hydrate.(6-9) One of the criteria for a compound to be a suspected human carcinogen is that the chemical causes cancer in more than one species of laboratory animal. DCA is the only one of the above mentioned TCE metabolites shown to cause liver cancer in both the rat and the mouse.(6,8) This indicates that DCA may be more of a threat than the other metabolites in causing cancer. The USEPA recognizes DCA as the most potentially harmful HAA in drinking water, as this is the only HAA5 with a maximum contaminant goal level (MCGL) of zero in drinking water and is the only HAA5 with the classification of probable human carcinogen.(10)

Analyzing for DCA has been quite problematic in environmental and biological samples. The most common method for analyzing DCA and other HAAs has been derivatization followed by gas chromatography (GC) analysis.(3,11-14) Two capillary electrophoresis (CE) methods also exist in which HAA samples are derivitized prior to analysis.(15,16) It has been found, however, that the strong acids used with the derivatizing reagents can convert TCA into DCA.(17) This

results in artificially high levels of DCA being reported. Several mass spectrometry methods have been reported for DCA.(18-24) However, many of these methods do not use HPLC.(19-22) Therefore, DCA was not isolated from the sample, resulting in a greater possibility of other chemicals in solution interfering with DCA analysis. Two of the LC/MS methods use sulfuric acid for pH adjustment of samples in order to protonate DCA. Also, two methods use ion pairing agents, which suppress ionization in the mass spectrometer. An HPLC method utilizing a conductivity detector has been described for the analysis of DCA in tap water.(25) Unfortunately, the limit of detection (LOD) of this method is 50 ng/mL, which is much higher than the levels of DCA that would be expected in drinking water. The USEPA is currently in the process of creating a new draft of the risk assessment for TCE which should take into account the impacts that DCA and TCA have on carcinogenicity. Therefore, it is important to have a sensitive, validated analytical method for quantitation of DCA. The use of LC/MS/MS allows DCA to be analyzed without derivitization.

Polar molecules in general produce sharp, reproducible peaks when run by HPLC. Reversed phase chromatography (RP-HPLC) using a C18 or C8 column is the most widely used procedure for the analysis of non-volatile compounds. However, small charged polar compounds such as DCA often elute in the solvent front when run by RP-HPLC because they lack affinity for the nonpolar stationary phase of most RP columns. Therefore, other RP columns must be used for the analysis of such compounds.

Hydrophilic interaction chromatography (HILIC) is a method by which the aqueous solvent, rather than the organic, determines how quickly the compound elutes. Columns containing a

polar end group such as an amino or silica column can be used in either HILIC or mixed HILIC-ion exchange chromatography.(26) In HILIC-ion exchange chromatography, retention is based on the affinity of the polar analyte for the charged end group on the column stationary phase. HILIC-ion exchange chromatography has been successfully utilized for many applications and is used in the method presented in this paper.(26) This paper describes the first HILIC-ion exchange chromatography method and the first LC/MS/MS method for the analysis of DCA.

EXPERIMENTAL

Chemicals and Reagents

HPLC grade acetonitrile and HPLC grade Optima Water were purchased from Fisher Scientific Company (Milwaukee, WI, USA). Ammonium formate salt and dichloroacetic acid, 99% were purchased from Aldrich (Fair Lawn, NJ, USA).

Method Development

The final method for DCA reported in this paper was determined after varying several experimental parameters. Several columns were investigated for use with this method. These included a Waters Xterra C18 column, a Phenomenex Synergi Max column, a Keystone Prizm column, and a Phenomenex Luna Amino column. Mobile phases investigated during method development included methanol/formic acid, methyl-tertbutyl ether (MTBE)/water, methanol/ammonium acetate, and acetonitrile (ACN)/ammonium formate. A range of buffer

concentrations (0-40 mM) were tested for the mobile phases listed. Two ion-pairing agents (tetraethylammonium hydroxide and triethylamine) and Dowex cation exchange resin were also investigated for use in this study.

Flow injection analysis of DCA was performed in order to optimize the mass spectrometer settings. All spectra obtained were in electrospray negative ionization (ESI^-) mode. The molecular ions $(M-H)^-$ of DCA with mass to charge ratio (m/z) 127 and 129 (chlorine isotopes) were observed, and source conditions were optimized to maximize these ions. Upon collisionally induced dissociation, major fragment peaks at m/z 83 and 85 were observed corresponding to the neutral loss of CO_2 (loss of 44). The collision energy as well as collision cell entrance and exit lenses were optimized to maximize this transition. The transition from m/z 127 to 83 was chosen as the transition to monitor because of its greater abundance. The ion at m/z 127 represents the more favored Cl^{35} isotopic species.

LC

An Agilent 1100 Series HPLC (Palo Alto, CA) was employed for this study. The HPLC was equipped with the following components: a degasser, a quaternary pump, an autosampler, and a thermostated column compartment. The HPLC column used in this study was a Phenomenex (Torrance, CA, USA) Luna Amino (150 x 2.1 mm, 5 μm particle size). A 4.0 x 2.0 mm Phenomenex Security Guard Amino guard column was also used. The column was kept at 25^o C for all experiments. The flow rate was 0.7 mL/min. Two mobile phases were utilized with the gradient run. The two components of the mobile phase were A (acetonitrile) and B (40 mM

ammonium formate made in HPLC grade water). The gradient run was as follows: 90 %A at time 0, 30 %A at 5 minutes, and 90 %A at 6 minutes. A re-equilibration period of 9 minutes then followed, making the total run time 15 minutes long. The injection volume was 10 μL, and the autosampler needle was rinsed with ACN between samples.

Mass Spectrometry

The mass spectrometer used in this study was a Micromass Quattro LC triple quadupole mass spectrometer fitted with a Z-spray source (Manchester, UK). The mass spectrometer was run in ESI^- using the MRM mode to monitor the transition from m/z 127 to 83. Settings for the capillary, cone, and extractor were respectively 0.50 kV, 15 V, and 2 V. The source temperature was set at 150^oC and the desolvation gas temperature was 350^oC. Gas flow rates were 1170 L/hr for the desolvation gas and 70 L/hr for the cone gas. The collision energy for dissociation was 9 eV and the entrance and exit lenses of the collision cell were set at -5 V and 35 V, respectively.

Sample Preparation

A 1 mg/mL stock solution of DCA was made in water each day samples were run. From this stock solution, a 10 μg/mL DCA solution was prepared by adding 10 μL of the 1 mg/mL stock solution to 990 μL of water. Samples for the calibration curves were made up in 60:40 ACN:water, the approximate percentages of ACN and water just prior to the time of DCA elution. A solution of 500 ng/mL was made by adding 300 μL of 10 ug/mL DCA into 5.7 mL of 60:40 ACN:water. This solution was used to spike all but three of the calibration curve samples.

A 50 ng/mL solution was made by adding 100 μL of the 500 ng/mL solution to 900 μL of 60:40 ACN:water. This solution was used to spike the three lowest calibration curve samples in order to decrease variability due to pipetting small sample volumes. An appropriate amount of either the 50 or 500 ng/mL DCA solution was added to 60:40 ACN to yield samples with DCA concentrations of 5, 10, 25, 50, 75, 150, 200, 250, 300, 400, and 500 ng/mL for use in a calibaration curve. Samples for validation were made in the same way and had concentrations of 5, 10, 15, 30, 100, and 500 ng/mL. Tap water samples (volume = 500 μL) were dried under vacuum and reconstituted in 100 μL of 60:40 ACN:water. This was done to have the tap water samples dissolved in the mobile phase composition and to concentrate the samples in order to improve sensitivity of the method.

Validation

On three separate days, an 11-point calibration curve was run along with 6 validation points (n = 5 for each validation point for one day, n = 15 for each validation point for all three days). In order to obtain the best fit for the data, a comparison was made between using linear calibration curves with no weighting and linear calibration curves with the following weightings: $1/x$, $1/x^2$, $1/y$, and $1/y^2$. Precision (% RSD) and accuracy (% Error) were calculated for the 6 calibration points. In order for a validation point to pass, both the precision and accuracy had to be less than 20% for the lower limit of quantitation (LLOQ) and less than 15% for all other points. The LLOQ was the lowest concentration sample which would pass validation. A 3:1 signal to noise ratio was the criteria used to determine the limit of detection (LOD).

Stability Studies

Autosampler stability was determined by pipetting a sample from the same solution into several autosampler vials. The samples were then injected every hour for 15 hours. Freeze/thaw stability was performed over 3 cycles.

Tap Water Samples

Drinking water samples were run to determine whether the method would give reliable results for a practical application. Tap water samples were obtained from several locations in Athens, GA, and areas surrounding Atlanta, GA. Three comparisons were made between samples: 1) amount of DCA in tap water vs. bottled water, 2) amount of DCA present from homes with a home filtration device versus those without a water filtration device, and 3) amount of DCA present in drinking water treated by different disinfection processes. Each sample had an n = 5.

RESULTS AND DISCUSSION

Results of Method Development

The Xterra and Synergi columns investigated for use in the analysis of DCA were found to be very sensitive to salt in samples. This was true to the extent that minute amounts of salt in samples (such as in tap water samples) shifted the peak for DCA to an earlier retention time and distorted the peak, resulting in area counts that were not reproducible. Higher salt concentrations

greatly distorted the peak such that the peak was not recognizable and eluted with the solvent front. Initial tests indicated that sodium was the main ion responsible for shifting the peak. A cation exchange resin was then used in an attempt to help improve the peak shape by replacing sodium ions with hydrogen ions. While this did help to some extent, too much salt remained in the samples. A Keystone Prism column was investigated, as this column had worked for the analysis of TCA (trichloroacetic acid), a compound that has similar problems with salt in samples.(27) However, this column was not capable of analyzing for DCA in the presence of sodium. A Phenomenex Luna Amino column was then chosen, because the amino column could be used in ion exchange mode, since the negatively charged DCA has an affinity for the positively charged amino groups. Sodium has no affinity for the amino groups, as both are positively charged, and therefore sodium should not be retained. An ACN:aqueous buffer mobile phase was chosen, because this combination of mobile phase has been shown to work well for HILIC separations of small polar compounds on amino columns.(26) Formate buffer was used because it is volatile. Forty mM was the optimum concentration of ammonium formate because this concentration resulted in the best peak shape with minimal ion suppression. Generally, as the concentration of buffer increased, the peak shape improved. However, high concentrations of buffer can result in ion suppression in the mass spectrometer. The optimum flow rate was determined to be 0.7 mL/min, because this resulted in the best peak shape as well as a faster run time. A chromatogram of DCA at the LLOQ and a blank chromatogram (60:40 ACN:HPLC grade water) are shown in Fig.1.

Validation

$1/y^2$ weighting was chosen because this type of weighing minimized the sum of the percent residuals, indicating the best fit. This type of weighting was also chosen because it gave more emphasis to the points at the lower end of the calibration curve, where the % error is often the highest. All calibration curves used in the validation of DCA had an R^2 value of greater than 0.99. For all 3 validation days, the calculated values for all 6 validation points corresponded well to the actual concentrations (Table 1). As shown in Tables 2 and 3, precision and accuracy values were less than 7.7 (%RSD) and 8.2 (%Error), respectively. Both of these values are well below 15%, the value required by the FDA for successful validation. The lower limit of quantitation (LLOQ) was found to be 5 ng/mL, and the limit of detection (LOD) was 1 ng/mL.

Stability Studies

DCA was stable in the autosampler over the duration of the stability study (15 hours). The compound was also stable over 3 freeze/thaw cycles.

Tap Water Samples

Calculated concentrations of DCA in tap water and bottled water samples are shown in Table 4. For water samples taken from an individual's residence, concentrations for both unfiltered tap water and tap water treated by a home filtration device are provided. All of the calculated DCA concentrations were shown to be reasonable, as tap water samples all showed agreement with

levels of DCA reported in the EPA's Safe Drinking Water Information System.(28) Bottled water samples contained much less DCA than tap water samples. This is not surprising, as DCA is commonly found in tap water as a disinfection by-product of chlorination. Bottled water may either not be treated with chlorine or there are so few tannins that DCA is not formed upon chlorination. It is interesting to note that in all cases except one (Covington, GA, home), household water filtration devices decreased the amount of DCA in the water sample. The one filter that did not remove DCA from tap water was old and in need of changing. Old filters can concentrate pollutants and when used too long can leach these pollutants into the water to be consumed. Also interesting to note is the fact that the filters used with the Covington, GA and East Athens, GA Home samples were pitcher-type water filtration devices and removed less than half of the DCA present in the tap water. In contrast, the South Athens Apartment and East Athens Apartment filtered water samples were both collected from home filtration devices that fit directly onto the water faucet. These filters removed the greatest percentage of DCA from the drinking water.

Concentrations of DCA were also compared among drinking water samples treated by each of the following disinfectant processes: chlorination, chlorine dioxide, and ozonation followed by chlorination. Concentrations for drinking water disinfected by each of the treatment processes can also be found in Table 4. There appeared to be no difference in DCA levels among the different drinking water treatments, based on limited sampling.

CONCLUSIONS

The method presented is the first LC/MS/MS analytical method for the determination of DCA and one of few for quantitation of small organic anions. The use of an amino column and ion exchange-HILIC chromatography allowed the small polar compound DCA to be analyzed without peak shifting due to salts in the sample. This method does not require the use of derivatization reagents shown to give inaccurate results for the analysis of DCA. The method uses small volumes (500 μL) of drinking water to detect low levels of DCA (LOQ = 5 ng/mL). This method is also the only known method for DCA analysis that has been validated using the criteria recommended by the US FDA. All precision and accuracy numbers for validation points were below 15%. The method was applied to a number of drinking water samples and the levels of DCA were determined to be between 12-28 ng/mL (ppb). Samples from bottled water contained low levels of DCA that were above the LOD, but below the LOQ of this method (roughly 1-1.5 ng/mL). Finally, the levels of DCA did not appear different when different water treatment strategies were employed.

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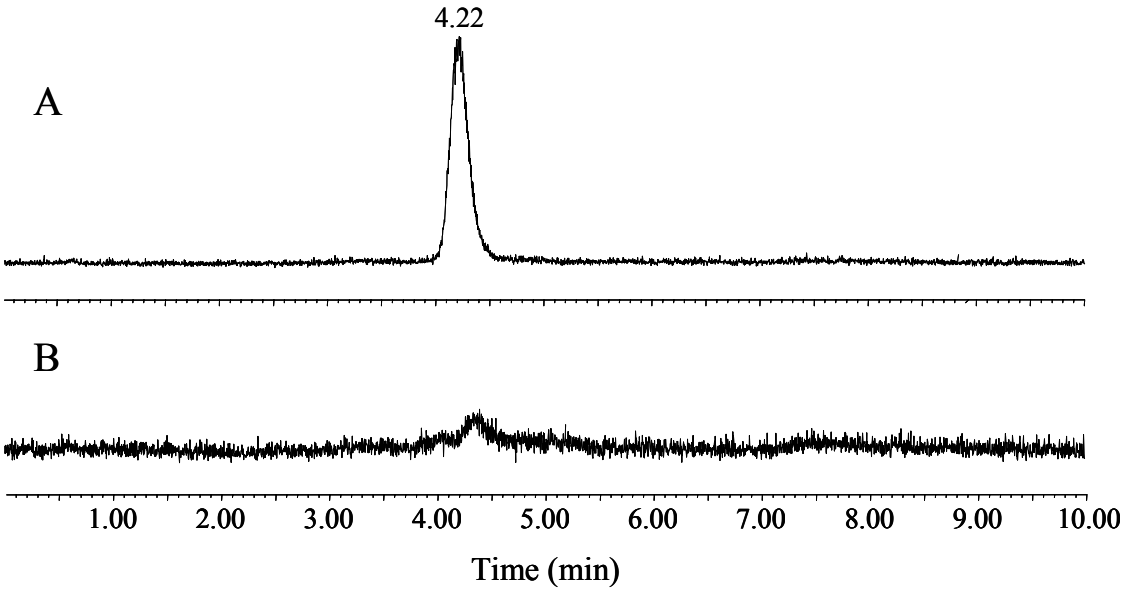

Figure 3.1 Chromatogram of DCA at A) LLOQ (5 ng/mL) and B) blank (60:40 ACN:water)

Table 3.1 Actual vs. Calculated DCA Concentrations on Validation Days 1-3

	Calculated DCA Concentration[a]			
Actual DCA Concentration (ng/mL)	Day 1 (n = 5)	Day 2 (n = 5)	Day 3 (n = 5)	Days 1-3 (n = 15)
5	4.6 ± 0.3	5.1 ± 0.2	5.1 ± 0.3	4.9 ± 0.4
10	10.3 ± 0.3	9.2 ± 0.2	10.0 ± 0.3	9.8 ± 0.5
15	15.3 ± 0.1	15.7 ± 0.4	15.9 ± 0.4	15.6 ± 0.4
30	29.8 ± 2.3	29.7 ± 0.7	30.9 ± 0.8	30.1 ± 1.4
100	107.2 ± 1.5	106.6 ± 2.1	104.6 ± 1.3	106.1 ± 6.1
500	460 ± 13.0	466.5 ± 3.3	460.0 ± 16.2	462.2 ±11.7

[a]All concentrations reported as x ± s, x = mean, s = standard deviation

Table 3.2 Accuracy Data for Method Validation

	% Error[a]			
DCA Concentration (ng/mL)	Day 1 (n = 5)	Day 2 (n = 5)	Day 3 (n = 5)	Days 1-3 (n = 15)
5	8.23	4.49	3.64	5.45
10	2.54	2.83	7.51	4.30
15	1.96	5.38	4.56	3.97
30	5.54	2.84	1.94	3.44
100	7.21	4.56	6.62	6.13
500	7.99	7.80	6.69	7.56

[a] % Error = Abs value [(A-O)/A]*100, A = actual concentration, O= observed concentration

Table 3.3 Precision Data for Method Validation

	% RSD[a]			
DCA Concentration (ng/mL)	Day 1 (n = 5)	Day 2 (n = 5)	Day 3 (n = 5)	Days 1-3 (n = 15)
5	5.57	4.77	5.56	5.30
10	3.13	2.52	3.48	3.05
15	0.96	2.87	2.46	2.10
30	7.68	2.28	0.89	3.61
100	1.41	2.00	1.23	1.55
500	2.82	0.71	3.52	2.34

[a] %RSD = (s/x)*100, s = standard deviation, x = mean

Table 3.4 Calculated Concentrations of DCA in Tap and Bottled Water

Drinking Water Source	Filtered[a] (ng/mL) n = 5	Unfiltered[a] (ng/mL) n = 5	Water[a] Treatment Type
East Athens, GA, Home	8.24 ± 0.01	14.2 ± 0.29	Chlorination
East Athens, GA, Apartment	ND[b]	25.2 ± 0.99	Chlorination
South Athens, GA, Apartment	ND[b]	12.2 ± 0.13	Chlorination
Covington, GA, Home	30.3 ± 1.08	28.1 ± 0.26	Chlorination
Vinings, GA, Business		13.3 ± 0.20	Chlorine Dioxide
Duluth, GA, Business		17.8 ± 0.29	Ozonation
Bottled Water Sample 1		ND[b]	
Bottled Water Sample 2		ND[b]	
Bottled Water Sample 3		ND[b]	
Bottled Water Sample 4		ND[b]	

[a]All concentrations reported as x ± s, x = mean, s = standard deviation

ND[b] = not detected because signal response was below lower limit of quantitation

CHAPTER 4

ANALYSIS OF DICHLOROACETIC ACID IN RAT BLOOD AND TISSUES BY HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

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ABSTRACT

Dichloroacetic acid (DCA) is a compound found in chlorinated drinking water. In addition, the compound is a metabolite of several halogenated solvents, including trichloroethylene (TCE) and perchloroethylene (PCE). Exposure to DCA is of concern, because high doses of the compound have been shown to cause cancer in laboratory animals. Dosages of TCE administered to animals in cancer studies are designed to elicit maximal DCA formation *in vivo*, whereas levels of DCA to which individuals are exposed to in drinking water are very low. Analysis of DCA in biological samples has been quite challenging. Derivitizing reagents commonly used to convert DCA to a more volatile form for GC analysis have been found to convert trichloroacetic acid (TCA), a major metabolite of TCE and PCE, to DCA. High performance liquid chromatography (HPLC) analysis does not require derivitization of DCA and can thus avoid this problem. However, the most popular stationary phases in HPLC columns do not retain small, polar compounds such as DCA well. The LC/MS/MS method described in this paper uses hydrophilic interaction liquid chromatography (HILIC), a type of chromatography that is able to retain these small, polar compounds. Method validation was performed using the United States Food and Drug Administration (USFDA) and International Conference on Harmonziation (ICH) Guidance for Industry: Bioanalytical Method Validation as a guide. Levels of DCA found in rats dosed with 2 g/kg TCE were 17.2 ng/mL (liver), 262.4 ng/mL (kidney), 175.1 ng/mL (lung), and 39.5 ng/mL (blood).

INTRODUCTION

Dichloroacetic acid (DCA) is a compound found in drinking water as a disinfection by-product of chlorination. In addition, DCA is a metabolite of several common solvents, including trichloroethylene (TCE) and perchloroethylene (PCE). TCE is also found in drinking water as a result of its widespread use as a metal degreaser and dry cleaning agent. Exposure to DCA is of concern, because high, chronic doses of the compound have been shown to cause liver cancer in mice and rats.[1-4] Individuals are exposed to DCA both directly through drinking water consumption and indirectly by metabolism of TCE and related solvents.[5-6] TCE is metabolized to DCA via the cytochrome P450 oxidative pathway.[7-8]

TCE, DCA, and TCA have been the subject of much discussion since the mid 1980s, due to the potential carcinogenicity of the three compounds in humans. TCE was found to cause liver, lung, or kidney cancer in at least one strain of rodent species. It has been determined that three metabolites of TCE (DCA, TCA, and chloral hydrate) are actually responsible for the observed liver carcinogenicity in mice.[1-4] DCA has also been found to cause kidney cancer in rats.[1,3,9] The ability to cause cancer in two sites in two species may indicate that DCA is more likely than TCA or CH to be a cancer risk to humans. The United States Environmental Protection Agency (USEPA) has set a maximum contaminant level (MCL) of 60 ng/mL total for the combination of 5 haloacetic acids (HAAs) in drinking water. The 5 regulated HAAs are DCA, monochloroacetic acid (MCA), trichloroacetic acid (TCA), monobromoacetic acid (MBA), and dibromoacetic acid (DBA). The USEPA recognizes exposure to DCA in drinking water as more of a threat than exposure to the other HAAs, as a minimum contaminant level goal

(MCLG) of 0 ng/mL has been set for DCA, while no MCLGs exist for the other 4 HAAs regulated in drinking water.[10]

Developing reliable analytical methods for DCA has been a difficult task. Several methods are available for the analysis of DCA and other HAAs in water.[10-35] Fewer methods are available for DCA in biological samples.[8,36-45] DCA is non-volatile, water soluble, and ionized in most solutions. Therefore, DCA would seem to be a good candidate for HPLC analysis. However, DCA is not retained well on reversed phase (RP) high performance liquid chromatography (HPLC) columns. In addition, DCA does not contain a strong chromophore. Therefore, it is not a good candidate for ultraviolet (UV) detection, which is commonly used with HPLC methods. The difficulty of analyzing DCA by HPLC is reflected by the fact that only five HPLC methods exist for DCA in water, and only one HPLC method is available for the analysis of DCA in biological samples (urine and plasma).[19-23,44] Most methods for DCA use a gas chromatograph (GC) for separation.[10-18] Because DCA is not volatile, it must be derivitized to a volatile form prior to GC analysis. The most common derivitizing reagent used in GC methods for DCA contains sulfuric acid. Derivitizing reagents containing sulfuric acid have been found to convert TCA to DCA.[41] This interconversion of chemical species has broad implications on making meaningful risk assessments of solvents such as TCE and PCE and the USEPA's goals of zero for DCA contamination of groundwater.

Recently, several columns have become available for the analysis of small polar compounds such as DCA. One type of chromatography that has been shown to work effectively for the retention and separation of small polar compounds is hydrophilic interaction liquid chromatography (HILIC).[23,46-51] Mobile phases used with HILIC typically consist of acetonitrile (ACN) and water. Increasing the amount of aqueous solvents, rather than organic compounds as

in reversed phase chromatography, results in faster elution of compounds. HILIC-ion exchange chromatography can also be performed. The mechanism of retention for HILIC-ion exchange is that the charged polar analyte has an affinity for the oppositely charged stationary phase on the column. Adding buffer to the mobile phase makes the analyte elute more quickly by displacing the analyte with another counter ion (buffer salt).

Validation of an analytical method is important in order to have confidence that the method is accurate and precise. The USFDA (United States Food and Drug Administration) and the ICH (International Conference on Harmonization) have developed guidelines for validating bioanalytical methods for industry.[52] These guidelines are very strict, because they are intended for validating methods for the analysis of pharmaceuticals. Incorrect measurements in such instances can result in patient morbidity and mortality. Validation of methods for compounds found in the environment (such as DCA) may not need to be as stringent as it would if the intended analyte was a pharmaceutical agent. Nonetheless, these guidelines for validation of bioanalytical methods are a good general guide. The method presented in this paper is the first HILIC-ion exchange chromatography for the analysis of DCA in rat blood and tissues and is the first method for DCA validated using these stringent bioanalytical method guidelines.

Due to the difficulty associated with analyzing DCA and issues of conversion of TCA to DCA, uncertainty remains as to whether DCA is actually formed from TCE *in vivo*. An analytical method for DCA that would not require derivitization would help to remove some of the uncertainty with metabolite species conversion. The goals of this study were to develop a validated method for the analysis of DCA in rat blood and tissues and to determine whether DCA is formed *in vivo* in rats dosed with TCE by use of a method that does not convert TCA to DCA.

EXPERIMENTAL

Chemicals and Reagents

HPLC-grade acetonitrile and HPLC-grade Optima Water were purchased from Fisher Scientific Company (Milwaukee, WI, USA). Ammonium formate salt (97%), dichloroacetic acid (99%), 2,2-dichloropropionic acid (Fluka), and difluoroacetic acid (DFA, 98%) were purchased from Aldrich (Fair Lawn, NJ, USA). Alkamuls EL 620 emulsifying agent was obtained from Rhodia (Cranbury, NJ, USA).

Consumable Supplies and Instrumentation

A Phenomenex (Torrance, CA, USA) Luna Amino HPLC column (150 x 2.0 mm, 5.0 μm) was used with a Phenomenex Security Guard amino guard column (4.0 mm L x 2.0 mm ID) in this study. Strata-X solid-phase extraction (SPE) tubes (200 mg/3mL) were also purchased from Phenomenex. An Agilent 1100 series HPLC consisting of a degasser, binary pump, autosampler, and thermostated column compartment was used in this study (Palo Alto, CA, USA). The mass spectrometer utilized for this work was a Waters Quattro Micro triple quadrupole mass spectrometer equipped with a Z-spray source and a built-in syringe pump (Manchester, UK). Tissue samples were homogenized with a Polytron PT 10-20-3500 homogenizer (Lucern, Switzerland). A Biofuge Pico microcentrifuge (Heraeus Instruments, Hanau, Germany) was used for sample centrifugation. Samples were evaporated to dryness in an Organomation Model 115 nitrogen bubbler (South Berlin, MA, USA). Adult male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA).

Method Development

The final procedure for analyzing DCA was determined after extensive method development. Several HPLC columns were investigated during development of the method. These included a Waters Xterra C18 column, a Phenomenex Synergi Max column, a Keystone Prizm column, and a Phenomenex Luna Amino column. Mobile phases investigated included methanol/formic acid, methyl-tertbutyl ether (MTBE)/water, methanol/ammonium acetate, and acetonitrile (ACN)/ammonium formate. Several buffer concentrations (0 - 40 mM) were evaluated. The suitability of tetraethylammonium hydroxide and triethylamine as ion-pairing agents and of a Dowex cation exchange resin for salt removal from samples was also investigated for use with this method. Several types of SPE cartridges were tested for the extraction of DCA from biological fluids. These included: ion-exchange (SAX, SCX, WAX), C18, amino, mixed phase (Screen-C, Oasis HLB, Strata-X, Strata-X-C), alumina, silica, and carbon based. Dichloropropionic acid (DPA) and difluoroacetic acid (DFA) were both assessed for their suitability as an internal standard. The effects of drying DCA in a vacuum centrifuge as compared to a nitrogen bubbler were also investigated.

Flow injection analysis of DCA was performed in order to optimize the mass spectrometer settings. All mass spectra were obtained in electrospray negative ionization (ESI^-) mode. The molecular ions $(M-H)^-$ of DCA with mass-to-charge ratio (m/z) 127 and 129 (chlorine isotopes) were observed, and source conditions were optimized to maximize these ions. Upon collision-induced dissociation, major fragment peaks at m/z 83 and 85 were observed corresponding to the neutral loss of CO_2 (loss of 44). The collision energy as well as collision cell entrance and exit lenses were optimized to maximize this transition. The transition from m/z 127 to 83 was chosen as the transition to monitor because of its greater abundance (Figure 1).

The ion at m/z 127 represents the more favored Cl^{35} isotopic species. Flow injection analysis was also performed in order to determine the mass of the precursor and fragment ions for the internal standard DFA in order to determine which mass transition to monitor. The most abundant parent peak for DFA was at m/z 95, while the most abundant peak after fragmentation of this peak was at m/z 51. Therefore, the transition from m/z 95→ 51, again representing the loss of CO_2 was monitored for DFA.

Sample Preparation

All tissue (lung, liver, and kidney) samples were homogenized with a volume of deionized water (in mL) equal to twice the weight (in g) of the tissue. The volume of tissue homogenate or whole blood used for samples was 180 µL. Samples used in calibration curves or as QC points for validation were spiked with 10 µL of the internal standard DFA (difluoroacetic acid, 500 ng/mL) and 10 µL of an appropriate concentration of DCA solution. All blanks and samples from the animal study were spiked with 10 µL of a 500 ng/mL aqueous DFA solution and 10 µL of HPLC grade water in 1.5 mL polypropylene centrifuge vials. Each sample was vortexed for 30 seconds after spiking with DCA and DFA. Protein precipitation was then accomplished by adding 600 µL of acetonitrile (ACN) to the 200-µL sample. Immediately after, each sample was vortexed again for 30 seconds. All samples were then centrifuged at 16000 x g for 5 minutes. The supernatant was removed and placed into a clean 1.5-mL centrifuge vial. Four hundred µL of 70:30 ACN:HPLC grade water were added to each sample to bring the total volume of each sample to approximately 1 mL. SPE was then performed on all samples.

Packing material in the SPE tubes was conditioned with 2 mL of ACN. Equilibration was achieved by adding 2 mL of HPLC-grade water to each SPE tube. Each 1 mL sample was

then loaded and allowed to pass through the SPE tubes under a light vacuum. Elution of DCA and DFA was achieved with 1 mL of HPLC-grade water. The sample loading and elution steps were collected in the same vial to minimize sample loss. Samples were evaporated to dryness in a nitrogen bubbler and then reconstituted in 100 μL of 70:30 ACN:HPLC-grade water. Each sample was subsequently filtered by centrifuging the sample in a Costar Spin-X (Corning Incorporated, Corning, NY, USA) filtration centrifuge tube. Seventy microliters of reconstituted sample were placed in an autosampler vial for HPLC/MS/MS analysis.

Conditions for Liquid Chromatography and Mass Spectrometry

The mobile phase used was 80:20 ACN:40 mM ammonium formate in HPLC grade water. Runs were 12 minutes in length, isocratic, and performed at a flow rate of 0.7 mL/min. The column was maintained at 25^{o} C for all experiments. The injection volume was 10 μL, and the autosampler needle was rinsed with ACN between samples.

The mass spectrometer was run in ESI negative ion mode using MRM to monitor the transition from m/z 127 → 83 for DCA and from m/z 95 → 51 for DFA. Settings for the capillary, cone, and extractor were, respectively, 0.20 kV, 20 V, and 1 V. The source temperature was set at 150^{o}C, and the desolvation gas temperature was 325^{o}C. Gas flow rates were 750 L/hr for the desolvation gas, and no cone gas was used. The collision energy for dissociation was 9 eV, and the entrance and exit lenses of the collision cell were set at -1 V and 30 V, respectively. Argon was used as the collision gas at a pressure of 1.0 x 10^{-4} torr.

Stock Solution and Calibration Samples

A 10 mg/mL stock solution of DCA was found to be stable in the freezer for six months and was therefore made once every three months. From this stock solution, a 100 μg/mL DCA solution was prepared by adding 10 μL of the 10 mg/mL stock solution to 990 μL of water. A solution of 10 μg/mL solution of DCA was made by adding 700 μL of an aqueous 100 μg/mL DCA solution to 6300 μL of HPLC-grade water. This solution was used to make solutions for spiking the four higher concentration samples on the calibration curve. A 1 μg/mL solution of DCA was prepared by adding 300 μL of an aqueous 10 μg/mL DCA solution to 2700 μL of HPLC-grade water. This solution was used to make solutions to spike samples in the lower half of the concentration curve in order to minimize error from pipetting. Appropriate amounts of water and either 10 μg/mL DCA or 1 μg/mL DCA solution were combined to make solutions to spike tissue samples with (20 times desired DCA concentration). A 10 mg/mL DFA solution was also prepared every three months. A 100 μg/mL DFA solution was prepared by adding 10 μL of 10 mg/mL DFA to 990 μL of HPLC-grade water. HPLC-grade water was used to perform a 10-fold dilution to yield a 10 μg/mL DFA solution. Samples for the calibration curves were made by adding 10 μL of DFA and 10 μL of the appropriate DCA solution to 180 μL of tissue homogenate or blood. This yielded calibration samples with concentrations of 10, 15, 25, 50, 100, 150, 200, and 250 ng/mL DCA and 500 ng/mL DFA.

Validation

All blood and tissue blanks used for validation were obtained from male Sprague-Dawley rats. An 8-point calibration curve and 4 validation points (10, 30, 75, and 250 ng/mL) were run on three days (n = 5 for each validation point for one day, n = 15 for each validation point for all

three days). Several types of weighting (no weighting, $1/x$, $1/x^2$, $1/y$, and $1/y^2$) were used in order to determine which best fit the data. Each type of weighting resulted in a linear calibration curve. Precision (expressed as % RSD) and accuracy (expressed as % Error) were calculated for the 4 validation points. In order for a validation point to pass, both the precision and accuracy had to be less than 20% for the lower limit of quantitation (LLOQ) and less than 15% for all other points.[52] The LLOQ was the lowest concentration sample which would pass validation and which had at least 3 times the response of the blank. A 3:1 signal to noise ratio was the criteria used to determine the limit of detection (LOD). Absolute and relative recovery and matrix effects were calculated for DCA samples (n = 5) at 10, 75, and 250 ng/mL and for 500 ng/mL DFA samples (n = 5) in each matrix. Absolute recovery was calculated as the response for DCA in a biological sample spiked before extraction divided by the response for an equal concentration DCA sample in 70:30 ACN:H_2O. Relative recovery was calculated by dividing the response for a DCA sample spiked before extraction by the response for an equal concentration DCA sample in the same matrix spiked after extraction. Matrix effects were calculated by dividing the response for a DCA sample in a biological matrix spiked after extraction by an equal concentration DCA sample in 70:30 ACN:H_2O.[53] Autosampler stability was determined by injecting 75 and 250 ng/mL DCA samples at 2 hour intervals over a 24-hour period. Freeze thaw stability was performed for 75 ng/mL DCA samples over 3 freeze-thaw cycles. The stability of DCA in biological samples was also assessed by spiking DCA and DFA in liver samples and then beginning sample preparation at different designated time intervals (0, 5, 10, 20, 40, and 60 minutes). The ability of freezing or heating samples to alter sample stability was determined both by freezing samples in liquid nitrogen and by heating samples.

RESULTS

Method Development

A Keystone Prism HPLC column, which has been reported in the literature to retain TCA, was investigated for DCA analysis with a variety of different mobile phases.[54] However, DCA was not retained with any mobile phase tested (MTBE/water, MeOH/water). Two similar HPLC columns investigated for use in this study (Waters Xterra and Phenomenex Synergi Max) retained DCA well in deionized water samples. However, the columns were very sensitive to the amount of salt in samples. This phenomenon has been observed in studies with TCA.[54] When tap water samples were run, the peak shifted to a retention time approximately 1 minute earlier. Increasing concentrations of NaCl in HPLC-grade water (from 0 to 10 mM) resulted in a shifting of peaks to earlier retention times. DCA-spiked water samples with NaCl concentrations of 100 and 1000 mM and all biological samples eluted near the void volume and had analyte peaks that were greatly distorted. Subsequent tests revealed that sodium was the salt most responsible for altering the retention behavior and peak shape. A cation exchange resin was used as a sample pretreatment in an attempt to remove sodium (replacing sodium with hydrogen). While this did improve peak shape, the chromatography was still not reproducible enough for quantitative analysis.

In order to solve the problem of salts (in particular cations) affecting the chromatography, an amino column was investigated for use in the analysis of DCA. With an amino column, the stationary phase is a positively charged amino group. Problematic cations should not be retained and, therefore, should pass through the column quickly and not affect the chromatography of compounds retained on the column. DCA is negatively charged and should be retained by a

chemical affinity for the positively charged amino groups. A mobile phase of ACN/aqueous buffer was tested for use with the amino column, because this type of mobile phase has been shown to work well for HILIC and HILIC-ion exchange applications for several small polar molecules.[46] DCA was retained with the amino column using an ACN/water mobile phase. Increasing the amount of water in the mobile phase elicited earlier elution of DCA, which is characteristic of the HILIC retention mechanism. A previously reported procedure used a phosphate buffer with an amino column for the retention of small polar compounds.[46] However, an ammonium formate buffer was chosen for use in the current study because formate, unlike buffers containing phosphate, is volatile and more suitable for use with LC/MS. Concentrations of ammonium formate buffer ranging from 0 – 40 mM were tested for use in the mobile phase. Increasing the concentration of the buffer resulted in earlier elution of DCA. This is typical of HILIC-ion exchange chromatography, as the increasing concentration of counter ions (formate) displace the anion analyte (DCA). Increasing the concentration of ammonium formate also resulted in sharper peaks. Ammonium formate concentration was increased until a desired peak shape and retention time were attained. A target retention time was one which was after 3.5 minutes, where ion suppression commonly occurs, but not so long that the time to analyze numerous samples becomes excessive. Once the target retention time and peak shape were attained, no more ammonium formate was added in order to minimize ion suppression due to the addition of buffers into the electrospray source. Representative chromatograms of DCA at the LLOQ (Figure 2), in blank samples (Figure 3), and in samples from animals dosed with TCE (Figure 4) are shown. Blank liver and kidney samples had low levels of endogenous DCA, which is likely present due to animals drinking tap water containing DCA. It should be noted that DCA elutes at approximately 4.4 minutes in tissue samples, but at 3.8 minutes in blood

samples. This difference in retention is related to the concentration of cations in the samples. While the retention times were different between blood and tissues, there is very little difference in the run-to-run retention times within a particular matrix. The addition of 500 mM NaCl to tissue samples can cause the retention times of these samples to reflect that of the blood samples.

Several different types of SPE cartridges were investigated for use in extraction of DCA. Phenomenex Strata-NH_2 SPE cartridges appeared to be the best to use, because over 90% of all DCA loaded on these cartridges was retained. In addition, peak shape in chromatograms from samples prepared on Strata-NH_2 was sharper than samples prepared with most of the other SPE cartridges. However, comparisons of samples containing DCA only versus samples with DCA and TCA passed through the Strata-NH_2 revealed that approximately 10% of the TCA present in samples was being converted to DCA on the SPE cartridge. Silica used as a support in the SPE cartridges was suspected to be causing the conversion. SPE cartridges containing bare silica supports converted approximately 4% of TCA in samples to DCA. Phenomenex Strata-X, Strata-X-C, and Oasis HLB all have polymeric supports rather than a silica support. Samples containing DCA only and samples with both DCA and TCA were prepared and compared to one another using these three types of SPE cartridges to determine whether TCA was converted to DCA on polymeric SPE tubes. No conversion was observed on any of the three polymeric SPE cartridges. Further testing was performed in order to determine which of these three SPE cartridges was best suited for DCA analysis. Strata-X-C cartridges retained less than 50% of DCA loaded on them, while Strata-X and Oasis HLB cartridge both retained approximately 50% of DCA. In order to minimize sample loss, both the sample loading and elution steps were collected in one vial. Therefore, the SPE cartridges are being used to retain interfering biological materials rather than being used in their traditional manner.

Samples prepared with Strata-X cartridges yielded better peak shape than samples prepared with Oasis HLB cartridges. As a result, Strata-X SPE cartridges were chosen for use in this method. Varying amounts (1 mL, 2 mL, 4 mL) of water were tested for the elution of DCA. One mL of water was sufficient to elute the remaining 50% of DCA off of the Strata-X cartridge. Performing a wash step after loading the sample was found to elute DCA. Therefore, no wash step was performed. After determining the best SPE cartridge and the optimum conditions for each step of SPE, calibration curves with DCA only and with DCA and TCA in biological matrix were prepared in order to determine whether conversion of TCA to DCA was occurring. Each calibration curve contained 5 points. Each calibration curve had points with equal concentrations of DCA. Samples in the calibration curve with DCA and TCA each contained a TCA concentration equal to one hundred times the DCA concentration. Both calibration curves had a linear response and each sample in each calibration curve had identical responses within the precision of the assay (Figure 5).

Difluoroacetic acid (DFA) and 2,2-dichloropropionic acid (DPA) were both tested for their suitability as an internal standard for DCA analysis. Both compounds were sufficiently similar to DCA to be detected under the same mass spectrometric conditions and to elute close to DCA. DPA, however, was found to suffer signal suppression with increasing DCA concentration. DFA was not suppressed at any DCA concentration and was chosen as the internal standard. Vacuum centrifugation and nitrogen bubbling, two methods of drying DCA samples, were compared to one another for their suitability in DCA analysis. Both methods for drying samples yielded similar responses in the mass spectrometer. However, it was found that DCA is semi-volatile at the lower pressures experienced in a vacuum centrifuge. Samples that were left in the centrifuge after reaching dryness began to lose DCA. No loss of DCA was

observed in the nitrogen bubbler before or after samples became dry. Therefore, the nitrogen bubbler was chosen as the method to dry samples.

A small amount of carryover (of DCA and DFA) was observed when a blank sample was injected after analysis of a sample spiked with DCA and DFA. Injection of a second blank showed no carryover. Therefore, a blank sample was injected between samples containing DCA and DFA in order to eliminate carryover. Some instrument drift was observed over the time that was needed for one sample set to run. A calibration curve was run at the beginning and end of each quantitated sample set in order to take drift into account in instrument response. Overall calibration curves were generated by averaging these two curves.

Validation

The type of weighting chosen for use in calibration curves was $1/y^2$. This type of weighting minimized the sum of the percent residuals, indicating the best fit for the data. The likely reason that $1/y^2$ weighting resulted in the best fit for the data is that this type of weighting gives more weight to points at the lower portion of the calibration curve, where the % Error is the highest. Precision and accuracy of DCA biological samples at all four validation concentrations and in all four matrices was less than 17.1 %RSD and 12.7 %Error, respectively (Table 1). These values are less than the %RSD and %Error required by the FDA and, therefore, pass validation. Relative recovery values for DCA in all matrices except liver were between 85 and 100%. Liver samples had relative recoveries of only 10–20 percent. Low recovery values for DCA in liver and high values for recovery of DCA in blood and other tissues has been observed in another study.[37] Absolute recovery values were high for kidney and lung samples (78% or greater), but liver and blood samples had low absolute recovery (9-28%). Higher

protein content in blood and liver, as compared to lung and kidney, may have led to lower recovery in these matrices. Calculated values for matrix effects were low in lung, liver, and kidney. Blood samples may have increased matrix effects due to the higher concentration of proteins and salts in blood. Samples from all matrices were stable in the autosampler over a 24-hour time period and in the freezer over 3 freeze/thaw cycles. The LLOQ was 10 ng/mL and the LOD was 5 ng/mL.

DCA was not stable in homogenized liver samples. Samples spiked with DCA and left to sit 5 minutes before beginning sample preparation yielded an approximately 50% response compared to samples spiked with DCA and processed immediately (Figure 6). When samples were spiked with DCA and allowed to sit for 10 minutes, there was a 75% drop in response compared to samples processed immediately. Samples allowed to sit for 20, 40, and 60 minutes before sample processing had approximately the same response, one-sixth that of samples prepared immediately after spiking. DFA, the internal standard, did not decease in concentration over time. It appeared that DCA was being further metabolized by the liver even following homogenization. Therefore, two methods to stop metabolism were tested. Both freezing samples in liquid nitrogen and heating samples at 65°C for 1 minute decreased the loss of DCA from samples. Heating appeared to be more effective in minimizing the loss of DCA. However, there was concern that while heating spiked homogenized samples appeared to work well, heating might increase the conversion of TCA to DCA. Therefore, freezing samples was ultimately chosen as the method to stop metabolism in tissue samples collected during animal studies.

Ion Suppression

Calculated ion suppression or enhancement values (based on matrix effect calculation) agreed well with ion suppression data obtained from injecting a blank matrix sample while infusing mobile phase and DCA into the mass spectrometer source. Blood was the only matrix in which appreciable ion suppression was calculated (Table 2). Chromatograms obtained from ion suppression studies showed that blood was the only matrix in which a decreased response was found at the retention time where DCA eluted. Liver, kidney, and lung samples had calculated ion enhancement and suppression values that, for the most part, were less than 20%. This is in agreement with the chromatograms collected for ion suppression studies with tissue blanks, as the signal at the retention time for DCA is close to that of the baseline. The amount of ion suppression from blood was significant and would be of substantial concern in human clinical studies where the composition of the matrix is highly variable. In the current study, however, matrix variation was minimal because all rats were from the same strain (Sprague-Dawley).

Animal Study

In order to determine whether it is possible to detect DCA in TCE-dosed animals and to show an application of the method, a small animal experiment was conducted. Sprague-Dawley rats (n = 3) were dosed orally with 2 g/kg of TCE as an aqueous Alkamuls emulsion. This dose, when given chronically, has previously been shown to cause kidney cancer in this strain of rat. Tissue samples were perfused with cold saline *in situ* in order to remove residual blood. Animals were sacrificed 2 hours after dosing with TCE. The results indicate that DCA is formed *in vivo* after dosing with TCE, as the DCA response was higher after dosing with TCE than in

the blank for all four matrices (Figure 7). Kidney samples had the highest levels of DCA (262.4 ng/mL). This may be the case, because the kidney is primarily responsible for DCA excretion. Lung and blood samples also exhibited higher levels of DCA two hours post-dosing compared to blank samples (175.1 ng/mL in lung, 39.5 ng/mL in blood). Liver samples had much lower levels (17.2 ng/mL), likely due to extensive metabolism of DCA by the liver.

CONCLUSIONS

Reliable analytical methods for DCA are essential in order to obtain accurate toxicokinetic and mechanistic information for use in cancer and noncancer risk assessments. Methods presented in the past have had serious issues with conversion of TCA to DCA, largely due to the derivitizating reagents. The method presented here does not use derivitizing reagents and has been shown to not convert TCA to DCA during sample preparation and handling. In addition, this method has been validated by the criteria recommended by the USFDA and the ICH. Precision and accuracy values (%RSD and %Error, respectively) are less than 15% in rat blood, liver, lung, and kidney. This method was used to demonstrate unequivocally that DCA is present in the blood and tissues of rats dosed with TCE. The presence of high levels of DCA in the kidney may suggest that DCA contributes to causation of kidney cancer observed in this strain of rat when dosed with TCE.

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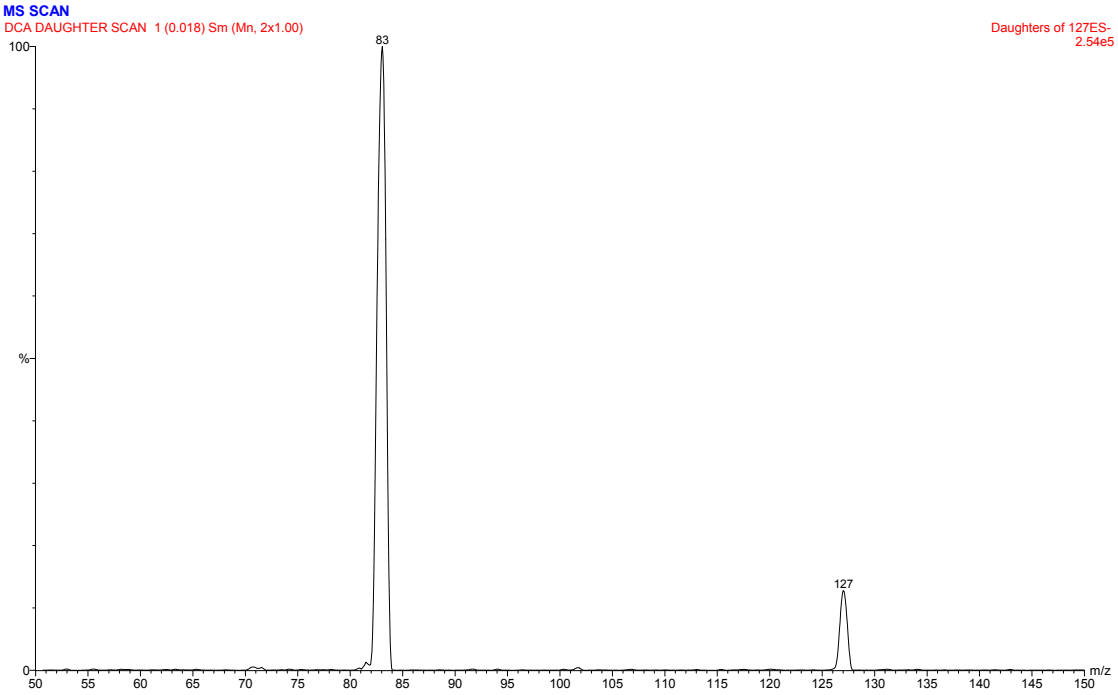

Figure 4.1 Product ion mass spectrum of DCA (m/z 127)

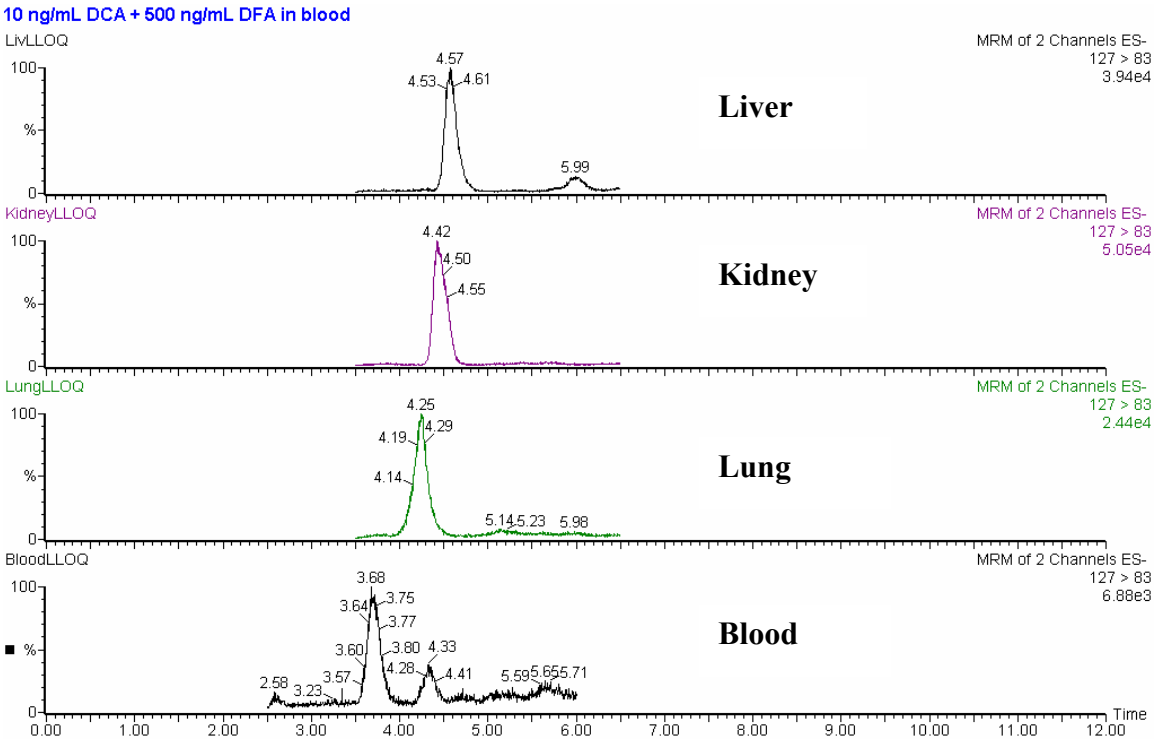

Figure 4.2 Chromatograms of DCA at LLOQ (10 ng/mL)

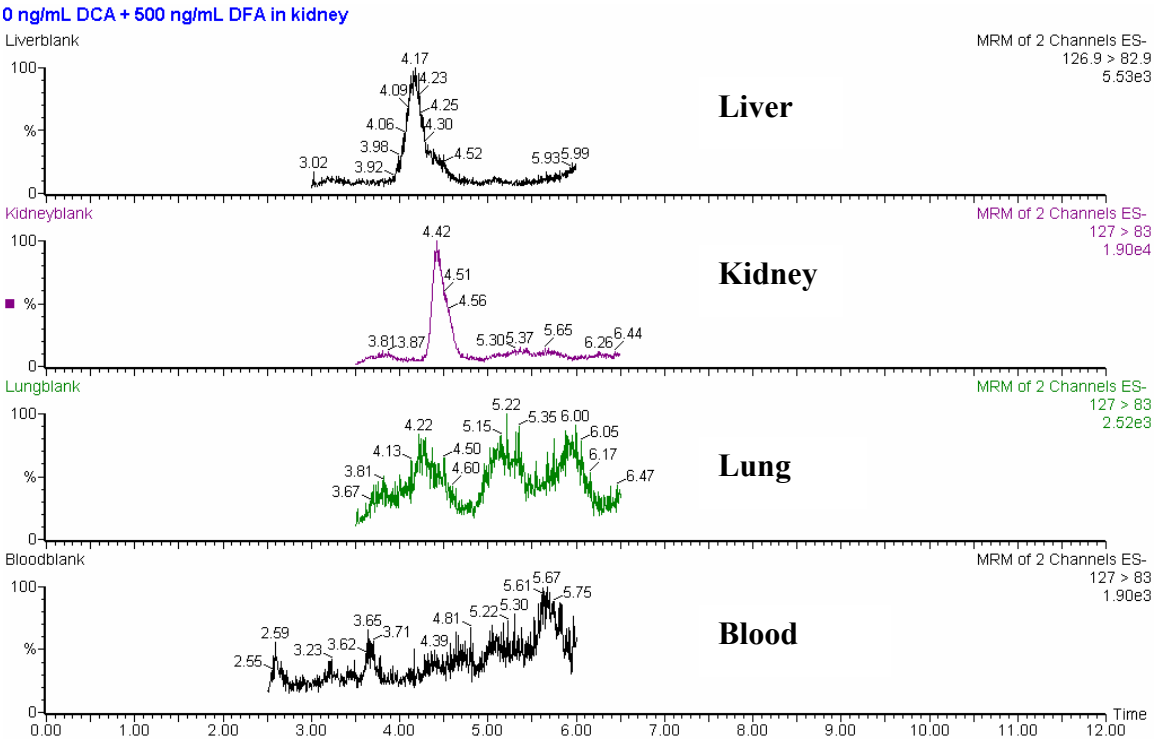

Figure 4.3 Chromatograms of blank samples

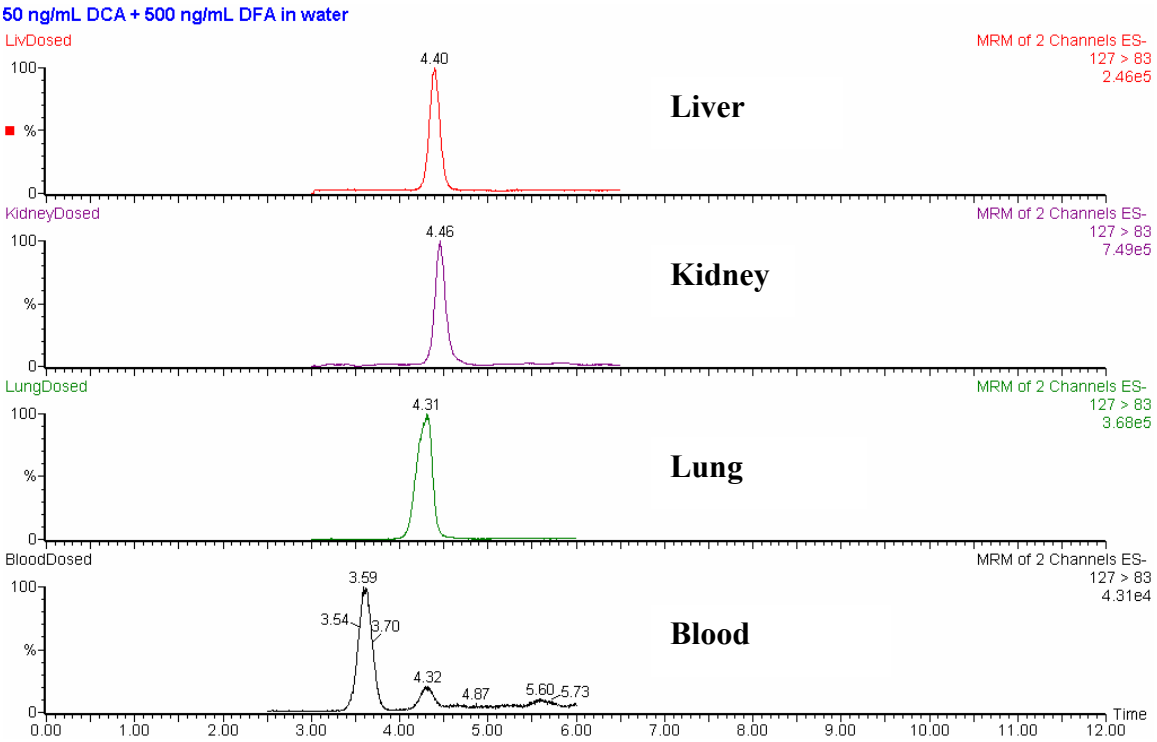

Figure 4.4 Chromatograms of DCA in rats dosed with 2 g/kg TCE

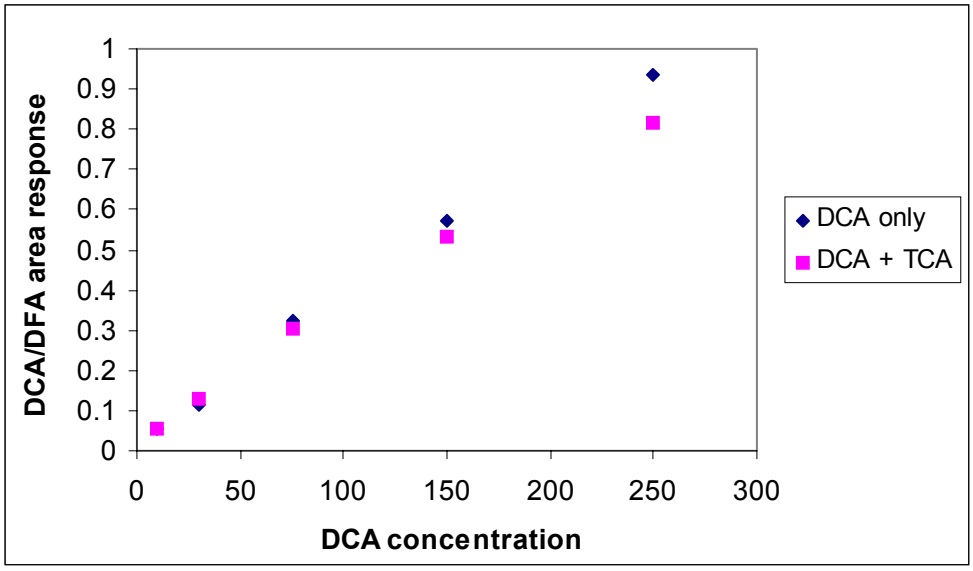

Figure 4.5 Comparison of calibration curves generated from samples containing DCA only and samples containing equal concentrations of DCA and TCA at a concentration equal to 100 times that of DCA

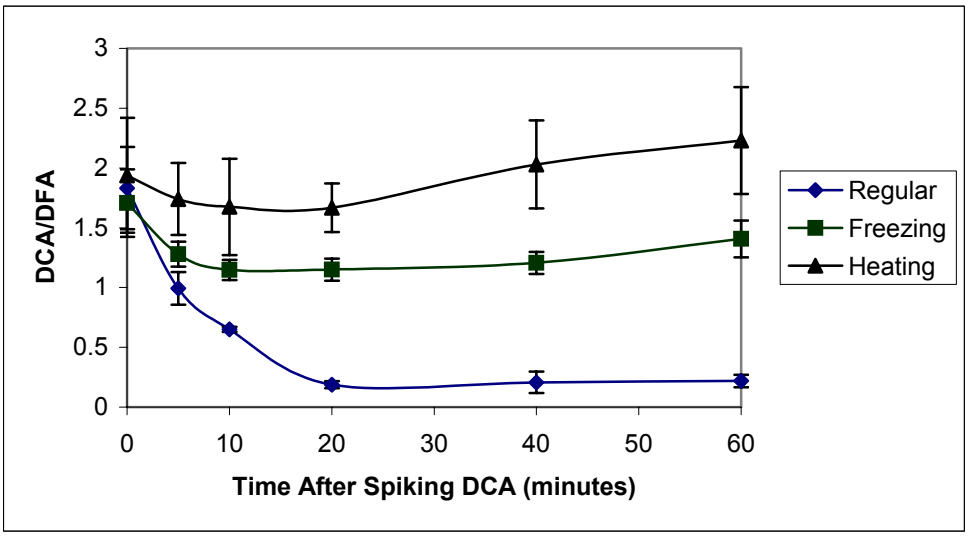

Figure 4.6 Stability of DCA in liver for samples prepared normally (at room temperature), frozen in liquid nitrogen, and heated at 65 °C for 1 minute

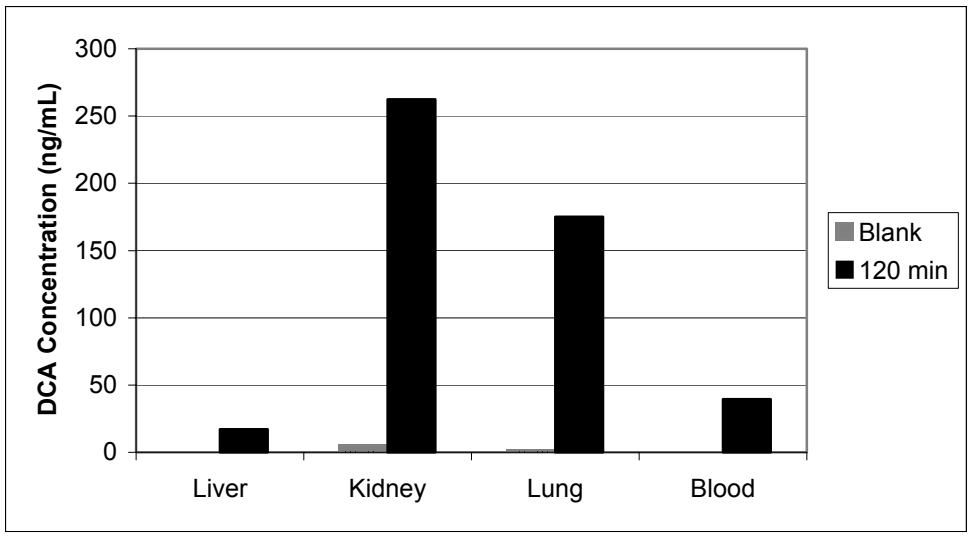

Figure 4.7 DCA Concentrations in rat blood and tissues, comparison of blank to samples, taken 120 minutes after dosing with TCE (2 g/kg).

Table 4.1 Precision and Accuracy Data for DCA Validation in Blood and Tissues

Matrix (n = 15)	Concentration (ng/mL) Actual	Calculated	% Error	%RSD
Liver	10	9.85 ± 0.85	6.52	8.63
	30	28.9 ± 1.8	5.85	6.25
	75	67.8 ± 5.9	10.5	8.70
	250	234.7 ± 24.7	10.1	10.5
Kidney	10	10.6 ± 1.80	12.7	17.1
	30	29.3 ± 4.1	11.7	7.32
	75	72.6 ± 5.3	6.29	7.32
	250	240.2 ± 23.3	9.10	9.69
Lung	10	10.1 ± 0.79	5.77	7.83
	30	29.1 ± 2.7	7.62	9.30
	75	73.4 ± 8.5	9.25	11.5
	250	237.1 ± 17.4	7.72	7.32
Blood	10	9.77 ± 0.52	4.39	5.30
	30	29.2 ± 1.25	3.88	4.28
	75	72.2 ± 3.11	11.6	4.31
	250	239.3 ± 7.37	4.27	3.10

Table 4.2 Precision and Accuracy Data for DCA Validation in Blood and Tissues

Matrix (n = 5)	Concentration (ng/mL)	Absolute Recovery Recovery (%)	Relative Recovery Recovery (%)	Matrix Effect (%)	Type of Effect
DCA					
Liver	10	11.5 ± 3.2	11.4 ± 2.3	101.3 ± 10.4	1.32% Enhancement
	75	9.17 ± 0.86	8.83 ± 0.97	103.9 ± 7.8	3.87% Enhancement
	250	19.6 ± 1.8	17.7 ± 1.9	110.7 ± 4.8	10.7% Enhancement
Kidney	10	125.5 ± 23.9	86.8 ± 11.3	135.1 ± 13.1	35.1% Enhancement
	75	100.4 ± 26.8	101.7 ± 25.8	98.7 ± 21.1	1.25% Suppression
	250	110.7 ± 26.8	99.3 ± 2.0	111.5 ± 26.7	11.5 % Enhancement
Lung	10	94.5 ± 17.2	85.3 ± 4.7	110.7 ± 13.2	10.7% Enhancement
	75	78.3 ± 23.2	94.6 ± 4.9	82.8 ± 24.3	17.2 % Suppression
	250	81.0 ± 16.3	84.2 ± 4.0	143.8 ± 23.4	43.8% Enhancement
Blood	10	22.3 ± 5.6	77.9 ± 12.6	28.6 ± 4.4	71.4 % Suppression
	75	21.2 ± 4.8	87.2 ± 7.8	24.3 ± 6.3	75.7 % Suppression
	250	27.5 ± 3.4	91.7 ± 7.4	30.0 ± 2.2	70.0 % Suppression
DFA					
Liver	500	20.3 ± 4.5	101.8 ± 12.0	19.9 ± 4.4	80.1% Suppression
Kidney	500	97.7 ± 23.7	97.8 ± 18.4	99.9 ± 18.4	0.10% Suppression
Lung	500	82.9 ± 12.3	88.2 ± 13.5	93.9 ± 13.5	6.01% Suppression
Blood	500	30.1 ± 2.5	84.2 ± 2.5	35.7 ± 2.5	64.3% Suppression

CONCLUSIONS

Trichloroethylene (TCE) and dichloroacetic acid (DCA) continue to be widely studied and discussed compounds, in part because of ongoing studies needed for new cancer and noncancer risk assessments. Difficulty in analyzing DCA, in particular, has slowed efforts to determine the extent of formation of DCA from TCE and the potential role of DCA in TCE-induced liver and kidney tumors. Issues with conversion of trichloroacetic acid (TCA), a quantitatively major metabolite of TCE, to DCA, a quantitatively minor metabolite of TCE, has caused uncertainty as to whether DCA is formed at all after exposure to TCE. Sensitive and reliable methods that do not require derivitization of samples should be able to assist in accurately addressing these toxicological questions.

Methods not requiring derivitization were developed for the analysis of TCE and DCA. TCE was measured by GC/MS, and DCA was analyzed by LC/MS/MS. This provides an alternative to the common practice of analyzing for TCE and its metabolites simultaneously by derivitizing biological samples with a reagent containing sulfuric acid. This has been shown to convert TCA to DCA. SPME-GC/MS conditions were optimized for the analysis of TCE. SPME is a relatively new technique that is growing in popularity due to its ease of use and minimal requirements for sample preparation. Reliable autosamplers for SPME are now widely available for use with GC systems, making SPME an even more attractive extraction technique by eliminating the need for analysts to manually inject each sample. SPME is likely to be used more in the future for TCE analysis, because this extraction method works for the compound and is relatively straightforward.

Methods are also presented for the analysis of DCA by HILIC-LC/MS/MS in drinking water and rat blood and tissues. To date, few HPLC methods exist for the analysis of DCA by HPLC. Hydrophilic interaction chromatography (HILIC) has been shown to be effective in the retention and separation of small, polar molecules such as DCA in several recently published papers. As HILIC grows in popularity, HPLC columns that are used with HILIC are becoming better known as alternatives to the traditional octadecyl reversed-phase columns. This trend will likely increase the number of HPLC applications for DCA. A validated method was presented for the analysis of DCA in drinking water. This method was shown to be effective for measuring DCA levels in bottled and tap water samples obtained from various locations in Georgia. The validated method presented in this paper for the analysis of DCA in rat blood and tissues has been proven not to involve the conversion of TCA to DCA. With this analysis, it has been possible to demonstrate that DCA is formed *in vivo* in rats dosed orally with TCE. This will likely make HPLC an attractive alternative to GC methods requiring derivitization for the analysis of DCA, particularly in toxicology studies.

In summary, methods for the analysis of TCE by SPME-GC/MS and DCA by HILIC-LC/MS/MS have been developed. These methods do not require derivitization of TCE or DCA and should, therefore, minimize concern about post sampling conversion of TCA to DCA by sulfuric acid in the derivitizing reagents commonly used in the analysis of TCE and its metabolites. DCA has been particularly difficult to analyze in the past. Use of the newly-developed analytical methods provide the assurance that the DCA in the kidneys, lungs, blood, and liver of rats dosed orally with 2 g/kg of TCE was actually derived from TCE.