QUANTITATIVE ANALYSIS AND TOXICOKINETICS OF TRICHLOROETHYLENE AND ITS METABOLITES IN RATS

by

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(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. Human exposure to TCE is primarily of concern due to its carcinogenicity in laboratory animals. The tumors evoked by TCE have been attributed to specific metabolites. Metabolites trichloroacetic acid (TCA) and dichloroacetic acid (DCA) are, in addition, frequently found in drinking water as byproducts of water chlorination. Doses of TCE typically given in animal studies are orders of magnitude higher than the levels to which individuals are exposed to environmentally. Nevertheless, there is concern on the part of EPA that even trace levels may present a cancer risk to humans. The human body possesses a number of defense mechanisms to protect against low-level toxic and mutagenic insults. First-pass, or presystemic elimination is one such process. Efforts to directly measure the capacity of first-pass elimination and metabolism of TCE under environmental exposure conditions have been hindered by difficulty with developing sufficiently sensitive, reliable analytical methods. Chapter 1 is the introduction and describes the scope of the dissertation. The optimization of headspace Solid Phase Microextraction (SPME) conditions for the determination of TCE in rat plasma is presented in

Chapter 2. The development and validation of a headspace SPME GC/MS method for the determination of TCE in rat blood and tissues is described in Chapter 3. A highly sensitive method using headspace SPME GC-NCIMS for the determination of TCE in biological samples is presented in Chapter 4. The LOQ for this NCI method is 10 times lower than the method described in Chapter 3. Chapter 5 presents two sensitive methods for the determination of TCE in rat adipose using SPME GC-EIMS and GC-NCIMS. In Chapter 6, an *in situ* derivatization/SPME GC-NCIMS method is validated for the determination of TCE metabolites including TCA, DCA and TCOH, in rat blood. All of the methods presented here are simple, rapid, sensitive and validated for specificity, linearity, accuracy and precision. Using the methods developed in previous chapters, the toxicokinetics and presystemic elimination of TCE in rats following environmentally-relevant exposures were characterized in Chapter 7.

INDEX WORDS: Trichloroethylene, TCE, Gas Chromatography, Mass Spectrometry, GC-MS, Headspace-Solid Phase Microextraction, HS-SPME, Trichloroacetic acid, TCA, Dichloroacetic acid, DCA, Trichloroethanol, TCOH, Negative Chemical Ionization, NCI, Presystemic elimination, First-pass, Toxicokinetics

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DEDICATION

This dissertation is dedicated with love to my family, my husband, Feng, my child, Kevin, and my parents, Xihai Liu and Wenqin Qi.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Trichloroethylene (TCE) is a halogenated volatile organic compound (VOC) that has been used extensively as a metal degreaser, chemical intermediate, anesthetic and drying cleaning agent. Much of the TCE used in the United States is released into the atmosphere from vapor degreasing operations. TCE contamination of ground and surface waters is largely attributed to industrial discharge of the chemical and leaching from hazardous waste sites (Bakke *et al.*, 2007; ATSDR, 1997; Wu and Schaum, 2000). As a result of its widespread use, TCE can be found in the groundwater in the proximity of more than 50% of the hazardous waste sites on the United States Environmental Protection Agency's (EPA) National Priorities List (Fay and Mumtaz, 1996). According to a survey by the National Water-Quality Assessment (NAWQA) program, 5% of wells throughout the United States have detectable levels of TCE, with concentrations ranging from 0.02 ug/L to 230 ug/L (Moran *et al.*, 2007). According to the Third National Health and Nutrition Examination (NHANES III), an estimated 10% of the US population has detectable levels of TCE in their blood (Ashley *et al.*, 1994; Churchill *et al.*, 2001). Human exposure to TCE in the environmental media is of concern, because very high, chronic doses of TCE have been found to be carcinogenic in laboratory animals (Bull, 2000; Rhomberg, 2000; EPA, 2001). Inhalation or ingestion of high doses of TCE for even a short time can produce central nervous system (CNS) depression, ranging from slight dizziness or sleepiness to unconsciousness and even death (ATSDR, 1997). Exposure to relatively high levels of trichloroethylene in air or water for a long period of time (years) can damage the liver and

kidney (Green *et al.*, 1998). Increased incidences of renal cell carcinoma have been reported in workers exposed occupationally, for years to high vapor levels of TCE (Lipworth *et al.*, 2006; Scott and Chiu, 2006; Raaschou-Nielsen *et al.*, 2003; Zhao *et al.*, 2005). Based on limited epidemiological data, TCE has been classified by the International Agency for Research on Cancer (IARC) and the National Toxicology Program (NTP) as a "probable human carcinogen" (National Toxicology Program, 2001; IARC, 1995).

The major pathway for TCE metabolism is oxidation via the cytochrome P450 system to chloral hydrate, and subsequently to trichloroethanol (TCOH), trichloroacetic acid (TCA) and dichloroacetic acid (DCA) (Bull, 2000). Chloral hydrate is a drug used as a sedative prior to surgery for adults, and for children who are undergoing a clinical procedure where they must remain still, such as magnetic resonance imaging (MRI) (Vade *et al.*, 1995; Keengwe *et al.*, 1999). Recent studies of TCOH showed that TCOH can induce nephrotoxicity in rats due to its conversion to formic acid and the resulting acidosis (Green *et al.*, 2003; Caldwell and Keshava, 2006). It has been determined that TCA and DCA are responsible for liver tumorigenesis in mice and/or rats (Caldwell and Keshava, 2006). TCA and DCA are, in addition, frequently found in drinking water as byproducts of water chlorination. Individuals are exposed to DCA and TCA, both directly via ingestion of chlorinated drinking water and indirectly by the metabolism of TCE and related solvents. The maximum contaminant level (MCL) for five haloacetic acids (including TCA and DCA) as set by EPA is 60 ng/mL. DCA is the only one of the five which is classified as a probable human carcinogen (EPA, 1998).

Determining the human relevance of animal carcinogenicity data and applying them to risk assessment of TCE and its metabolites has been a source of controversy since the mid 1980s. The EPA is currently reviewing toxicity and toxicokinetic data on TCE and its metabolites in

order to revise its cancer and noncancer risk assessments of the chemical. Different aspects of analytical methods and toxicokinetics of TCE and its metabolites are addressed here.

PART I ANALYTICAL METHODS

1. Separation techniques

Separation techniques are typically combined with some type of detection for the analysis of TCE, DCA, TCA and TCOH. This section discusses the main types of separation procedures including gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) used in the analysis of TCE and its metabolites.

a) 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 mass spectrometry (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). Several methods also exist for the analysis of TCE in biological samples (Brown *et al.*, 2003b; Muralidhara and Bruckner, 1999; Koppen *et al.*, 1988; Ashley *et al.*, 1994; Dehon *et al.*, 2000; Poli *et al.*, 2005; Xu *et al.*, 1996; Dixon *et al.* 2005; Wu *et al.*, 2002).

For the analysis of TCE metabolites, GC is by far the most commonly used separation technique. DCA and TCA have p*K*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 derivatizing the compounds or by decreasing the pH to 0.5 in order to neutralize the anions. Derivatization reagents such as diazomethane (Kristiansen *et al.*, 1996), dimethyl sulfate (Ketcha *et al.*, 1996), 3-methyl-1-tolytriazene (Humbert *et al.*, 1994), BF_3-MeOH (Yan *et al.*, 1997) and acidic methanol (Song *et al.*, 2003; Muralidhara and Bruckner, 1999; Wang and Wong, 2005) are often applied to acidic analytes. A mixture of sulfuric acid and methanol is one of the most common derivatizing agents, and no poisonous or explosive chemicals are involved. One study, however, found that using sulfuric acid as a derivatizing reagent resulted in conversion of TCA to DCA (Ketcha *et al.*, 1996).

b) HPLC

HPLC is used far less often than GC in the analysis of TCE and its metabolites. TCE and TCOH are volatile and are therefore much better suited for GC analysis. Gupta (1990) reported a method for the determination of TCOH in plasma by a high performance liquid chromatography-ultraviolet (HPLC-UV) method that required a complicated derivatization procedure prior to analysis. DCA and TCA, however, would be expected to be better adapted to HPLC analysis than GC analysis due to their non-volatile nature. The type of chromatography most commonly used with HPLC is reversed-phase (RP) chromatography. TCA and DCA are small and charged polar molecules in most solutions; therefore, they are not retained easily on the nonpolar stationary phase in the RP HPLC columns. Kuklenyik *et al*. (2002) were able to retain TCA in human urine samples using reversed phase chromatography with tandem mass spectrometry (RP-HPLC-MS/MS). TCA is extracted from the urine using solid-phase extraction (SPE), separated from other extract components by reversed-phase HPLC, and analyzed by negative ion

electrospray ionization isotope dilution-MS/MS using a multiple reaction monitoring experiment. The LOD and LOQ were calculated and reported as 0.5 and 1.7 ng/mL, respectively.

Due to the charged character of TCA and DCA, ion-pair and ion-exchange chromatography are also employed for their analysis in drinking water, plasma or urine samples (Loos and Barcelo, 2001; Narayanan *et al.*, 1999; Takino *et al.*, 2000) with limits of detection in low µg L^{-1} levels. With ion-pair chromatography, an ion-pairing agent is added to the mobile phase. The organic portion of the molecule associates with the stationary phase, and the charged portion of the compound is exposed. This allows polar and charged compounds to be retained by interation with the exposed polar portion of the ion-pair agent. 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.

Another type of chromatography, hydrophilic interaction liquid chromatography (HILIC), is also commonly used for highly polar compounds. HILIC is a variation of normal-phase chromatography without the disadvantages of using solvents that are immiscible with water. With HILIC, the stationary phase is a polar material such as silica, cyano, amino, diol, etc. The mobile phase is highly organic (> 80%) with a small amount of an aqueous/polar solvent. HILIC-ion exchange chromatography, in which there is the additional retention mechanism of ion exchange, can also be performed. Dixon *et al*. (2004; 2005) used HILIC-ion exhange chromatography with tandem mass spectrometry for the analysis of DCA in drinking water and rat tissues. An amino column was used in these studies. The limit of quantitation (LOQ) was reported as 5 ng/mL in water and 10 ng/mL in rat blood and tissues, respectively.

c) Capillary electrophoresis (CE)

CE is another technique not as widely used as GC for the analysis of TCE metabolites. Only charged compounds can be measured by CE, such as TCA and DCA. In traditional electrophoresis, electrically charged analytes move in a conductive liquid medium under the influence of an electric field. The technique of CE was designed to separate species based on their size to charge ratio in the interior of a small capillary filled with an electrolyte. Ahrer and Buchberger (1999) developed a method for the analysis of nine haloacetic acids (HAAs) in water samples by CE-MS. Liquid–liquid extraction was performed in order to extract HAAs from water samples. The linear ranges for DCA and TCA were 0.1–5 µg/mL and 0.2–3 µg/mL, respectively. When analyzing 30-mL water samples (in which concentration steps were performed) detection limits were 0.3 ng/mL for DCA and 0.5 ng/mL for TCA. The advantage of CE for the measurement of DCA and TCA is that no derivatization step is required. The disadvantage of using CE are high LOQs unless a very large sample is available.

2. Sample pretreatment techniques

Before analyzing TCE and its metabolites, it is often necessary to extract the compounds from the biological matrix. Sample pretreatment is a critical step in the analysis of compounds from biological fluids. There are several extraction methods including liquid-liquid extraction (LLE) and solid phase extraction (SPE) for extracting the compounds from various matrices. Because of the special properties of volatile organic compoundVOCs, some extraction techniques available include purge and trap (PT), headspace (HS) and solid-phase microextraction (SPME).

a) Liquid-liquid extraction

Liquid-liquid extraction, also called solvent extraction, is a process that allows the separation of two or more components due to their unequal solubilities in two immiscible liquid phases. This extraction technique is particularly useful for TCE due to its high lipid solubility. An organic solvent is simply added to a drinking water sample or tissue homogenates, and TCE partitions into the organic phase, which is collected (Brown, *et al.*, 2003a; 2003b). Although TCA and DCA are hydrophilic compounds, many methods exit involving liquid-liquid extraction from water or biological matrices into an organic phase, followed by derivatization and analysis of the corresponding methyl esters by GC (EPA, 1990; EPA, 1995; Schmitt, 2002; Song JZ and Ho JW, 2003; Humbert L *et al.*, 1994; Yan Z *et al.*, 1997). LLE is simple and involves solvents that are usually readily available and inexpensive. However, it has been found that many solvents used in LLE are contaminated with TCE (Brown *et al.*, 2003a). LLE also involves a number of additional sample preparation steps which results in increased cost, time, potential error and decreased sensitivity of the analysis.

b) Solid-phase extraction

Solid-phase extraction (SPE) is a method that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a solution. It is usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the amount of analyte(s) in the sample. The general procedure is to load a solution onto the SPE phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube. SPE is used primarily for non–volatile, 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 DCA and TCA in water and biological samples using SPE (Loos and Barcelo, 2001; Sarzanini *et al.*, 1999; Kuklenyik *et al.*, 2002; Calafat *et al.*, 2003; Dixon *et al*., 2005).

c) Headspace

The 'headspace' is the gas space in a sample vial above the sample. Headspace analysis is used for the analysis of volatiles and semi-volatile organics in solid, liquid and gas samples. Complex sample matrices, which require sample extraction or preparation, or are difficult to analyze directly, are ideal candidates for headspace, since they can be placed directly into a vial with little or no preparation. Sealed vials containing samples are held at a constant temperature for a known period. After the volatile agent has reached equilibrium between the gas and the sample phase, a small volume of the headspace (0.025–1.0 mL) is injected onto a GC column. Muralidhara and Bruckner (1999) report a headspace GC-ECD method with a LOQ of 50 ng/ml for the measurement of TCE and its metabolites in blood. Another HS-GC-ECD method was described in Koppen's paper for the determination of TCE metabolites in rat liver homogenates (Koppen *et al.*, 1988). This method was based on selective thermal conversion of chloral hydrate and TCA into chloroform. Traditional headspace GC analysis is not sensitive enough, because this technique does not involve sample pre-concentration.

d) Purge and Trap

Similar to static headspace, purge and trap techniques (also called dynamic headspace analysis) allow a sample to be analyzed using the gas phase above the sample. With purge and trap, the sample is continuously purged with an inert gas (usually helium) and the volatile components collected on a sorbent trap. The purging gas can either be bubbled through, or swept over the sample. After the sample has been purged, the trap is thermally desorbed, and the

volatile components are injected onto a GC column. Ashley *et al.* (1994) reported a method using purge and trap concentrator in conjunction with GC-MS for the determination of parts per trillion levels of 32 VOCs, including TCE, in the blood of nonoccupationally-exposed humans. A detection limit of 0.01 ng/mL was found for TCE. Zoccolillo *et al.* (2005) used an analytical system composed of a purge-and-trap injection system coupled to gas chromatography with mass spectrometric detection (PTI-GC–MS) specific for the analysis of TCE and some other volatile chlorinated hydrocarbons and trihalomethanes in water. A sample volume of 10 mL water was purged with a stream of helium for 10 min at ambient temperature. The purge flow containing volatiles was passed through a cold trap at a temperature of −100 ºC. At the end of the purge, flash heating of the cold trap (200 ºC) injected the substances into the capillary GC. Detection limits of this method were very low, ranging from μg/L to ng/L. The LOQ for TCE was 1 ng/L. Johns *et al.* (2005) described a method for the analysis of TCA in urine using dynamic headspace GC-MS. Samples were analyzed using the soil module of a modified purge and trap autosampler to facilitate the use of disposable purging vessels. The LOQ for TCA was 9 ng/mL. Purge and trap analysis is sensitive but time consuming and requires a special apparatus to maintain low temperatures.

e) Solid-phase Microextraction

Solid phase microextraction (SPME) is an innovative, solvent-free technology that is fast, economical, and versatile. With this method, a coated fiber is exposed to the volatilized chemical in the sample's headspace. The analyte partitions from the sample matrix to the coating. The fiber bearing the concentrated analyte is then transferred to the analytical instrument where desorption, separation, and quantification of the extracted analyte takes place (Alpendurada, 2000). SPME has several advantages over classical GC techniques. SPME can

pre-concentrate the samples due to the high affinity of the analyte for the fiber coating. Potentially contaminated organic solvents commonly used for extracting volatile analytes from biological samples are not needed. Therefore with SPME, sample handling is minimized. When a biological sample is taken, it is simply placed into an autosampler vial and capped until analysis. This limited sample preparation helps to minimize the loss of volatile analytes and reduces potential error. Furthermore, by using headspace SPME methods, the fiber is only exposed in the vapour phase above the sample. The analytes need to be transferred through the barrier of the air before they reach the coating. This modification can protect the fiber coating from damage by high molecular mass and other non-volatile interferences present in the sample matrix.

Several methods have been published describing the use of SPME for analysis of TCE and its metabolites. Some of them are focused on the measurement of TCE, TCA or DCA in environmental media, such as air, drinking water and soil (Pastore *et al.*, 2005; Larroque *et al.*, 2006; Bocchini *et al.*, 1999; Aikawa *et al.*, 1997; Sarrion *et al.*, 1999). Quantitation of chemicals in a biological matrix is difficult due to matrix effects. Dehon *et al.* (2000) reported a headspace SPME GC-MS method for determination of TCE and its metabolites TCA and TCOH in a human fatality. Relatively large volumes of samples were required for their method. Optimization and validation of the method were not described in this paper. Poli *et al.* (2005) developed a sensitive method for the determination of volatile chlorinated hydrocarbons, including TCE, in human urine samples using HS-SPME GC-MS. The highest extraction efficiency was obtained when sampling was performed at room temperature (22 ºC), from samples saturated with salt and under agitation. Linearity of the HS-SPME-GC-MS method was established over four orders of magnitude. The limit of detection was 0.01 μg/L for TCE. Two ml of urine sample was used in their method. This large a sample volume cannot be obtained

from small animals where multiple sampling is necessary for toxicokinetic studies. Xu *et al.* (1996) reported a GC-MS method coupled with SPME for the determination of TCE in environmental biological samples, such as the microcosm of a cell. Their method's detection limit was 5 μg/L, but complete immersion of the fiber in the biological samples substantially shortens the life of the fiber coating. Dixon *et al.* (2005) developed a SPME procedure for analysis of TCE in rat blood, plasma, liver, kidney, and lung with a LOQ of 5 ng/mL. For all biological specimens, placing the fiber in the sample headspace for 5 minutes for extraction of TCE gave maximal detector response. The length of time that the sample was heated had no effect on the TCE response. This method was optimized but not validated. Wu *et al.* (2002) reported a SPME-GC-ECD method for the analysis of TCA, DCA and several other haloacetic acids. Derivatization 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.

3. Detectors

Several types of detectors are available for the determination of TCE and its metabolites. Some of these include mass spectrometers (MS), electron capture detectors (ECD), flame ionization detectors (FID) and ultraviolet detectors (UV). Mass spectrometers are commonly used with GC, 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. TCE and its metabolites have only one double bond which absorbs UV lights. Therefore they are not ideal candidates for methods employing UV detection. Poor UV

absorption and high detection limits are likely the reason for the scarcity of HPLC-UV methods in the literature. Therefore, only MS, ECD and FID are included in this section.

a) Mass Spectrometers

Mass spectrometers are commonly used as detectors for GC and LC applications. MS is an analytical technique that measures the mass-to-charge ratio of charged particles. This detector has outstanding sensitivity, stability, specificity and dynamic range. All mass spectrometers have three basic components: (1) an ionization source, (2) a mass analyzer and (3) a detector. For LC applications, the most commonly used means of sample introduction is electrospray ionization (ESI). Most published LC-MS methods for the determination of TCA and DCA use ESI as the ion source (Ells *et al.*, 2000; Kuklenyik *et al.*, 2002; Dixon *et al.*, 2004; 2005; Calafat *et al.*, 2003; Hashimoto and Otsuli, 1998; Takino *et al.*, 2000; Loos and Barcelo, 2001). Two "classical" ionization methods, electron ionization (EI) and chemical ionization (CI), are commonly used for the analysis of low-mass, volatile, thermally-stable organic compounds, such as TCE and TCOH, when coupled with GC. With EI, ions are generated by bombarding gas phase molecules with high energy electrons. Most GC-MS methods published for the determination of TCE and its metabolites in biological samples involved electron ionization (Song and Ho, 2003; Dehon *et al.*, 2000; Yan *et al.*, 1997; 1999; Ashley *et al.*, 1994; Brown *et al.*, 2003b; Xu *et al.*, 1996; Poli *et al.*, 2005; Johns *et al.*, 2005; Dixon *et al*., 2005; Zoccolillo *et al.*, 2005). CI is an alternative to EI. CI uses a reagent ion to react with the analyte molecules to form ions by either a proton or hydride transfer. For those compounds containing acidic groups or electronegative elements (especially halogens), negative chemical ionization (NCI) is used for increased sensitivity and selectivity (Mateo-Vivaracho *et al.*, 2006; Lai *et al.*, 2006). With NCI, thermal electrons are first created from a heated metal filament. When molecules interact with

these thermal electrons, an electron capture process occurs to generate $M^{-\cdot}$ molecular ions. The sensitivity of procedures using NCI is often 3- to 100-fold greater than ECD methods (Kontsas and Pekari, 2003), and 2-100 times more sensitive than EI-MS (Xu *et al.*, 2006; Jonsson *et al.*, 2006; Jia *et al.*, 2003). Jia *et al.* (2003) developed a GC-NCI-MS method using pentafluorobenzyl bromide (PFBBr) as a derivatizing reagent for quantifying nine haloacetic acids (including TCA and DCA) present in plasma, urine, and water at picogram per milliliter levels. Liquid-liquid extraction using methyl tertbutyl ether and diethyl ether were performed to extract the HAAs from water or biological samples. The detection limits of HAAs in plasma, urine, and water were 25 ng/L.

b) Electron capture detection

Electron capture detectors (ECDs) are commonly used with GC methods. The ECD uses a radioactive Beta particle (electrons) emitter -- a typical source contains a metal foil holding 10 millicuries of Nickel-63. The electrons formed are attracted to a positively charged anode, generating a steady current. As the sample is carried into the detector by a stream of nitrogen or a 5% methane, 95% argon mixture, analyte molecules capture the electrons and reduce the current between the collector anode and a cathode. The analyte concentration is thus proportional to the degree of electron capture, and this detector is particularly sensitive to compounds containing electronegative groups, making it very sensitive for analysis of halogenated compounds such as TCE, DCA, TCA and TCOH. In many cases, the ECD detector is the most sensitive detector for the analysis of halogenated compounds. The USEPA uses GC-ECD for the analysis of TCE and its metabolites (Munch and Hautman, 1995; Munch, 1995; Domino *et al.*, 2003). In addition, some of the GC methods discussed in this paper employ ECD detection (Muralidhara and Bruckner, 1999; Kim *et al.*, 1999; Wu *et al.*, 2002; Ketcha *et al.*, 1996).

c) Flame ionization detection

Flame ionization detection (FID) is another type of detection commonly used with GC analysis. With 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. FID is a universal detector. Except for a very few organic compounds (e.g. carbon monoxide, etc.), FID detects all carbon-containing compounds. FID has a very wide dynamic range, but a relatively low sensitivity as compared with other detectors for the analysis of TCE and its metabolites, such as MS and ECD. 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.

PART II TOXICOKINETICS

In 2001, the EPA proposed the adoption of a regulatory approach which assumes that trace levels of TCE present a cancer risk. This approach does not take into account the toxicokinetic behavior of low doses of TCE, nor the body's capacity to deal with low levels of environmental contaminants.

The human body possesses a number of defense mechanisms to protect against low-level toxic and mutagenic insults. These mechanisms include presystemic elimination, metabolic detoxification, DNA repair, death of mutated cells, apoptosis, destruction of mutated cells by the immune system, and the action of tumor suppressor genes. The extremely high doses of TCE and other VOCs administered in rodent cancer studies "overwhelm" these protective processes, kill cells, exceed the capacity of tissue repair, and can thereby cause tumors in some strains of mice and rats. A substantial amount of TCE and other well-metabolized VOCs may be removed from

the portal venous blood by presystemic, or first-pass elimination. Drugs and chemicals absorbed from the gastrointestinal tract must first pass though the portal blood into the liver and on to the lungs, before entering arterial circulation and being transported to tissues throughout the body (Gibaldi and Perrier, 1982; Routledge *et al.*, 1979). In 1981, Andersen conducted a series of studies of the systemic uptake of inhaled halocarbons by rats. He concluded that the liver was capable of removing virtually all of a halocarbon presented to it, so long as the dose did not saturate (i.e., exceed the capacity of) metabolism. Only Lee *et al.* (1996) have provided empirical data to support this important postulate. Their experimental results demonstrated that the liver and lungs, acting in concert, can remove a significant proportion of moderate oral doses of TCE. Liver first-pass elimination was found to be inversely related to dose, with as much as 60% of the lowest doses removed. This phenomenon could result not only from metabolic saturation, but from suicidal destruction of cytochromes P450 and hepatocellular injury as well (Lee *et al.*, 2000a). Direct solvent injury of liver cell membranes played a modest role in reducing the liver's capacity to metabolize high TCE doses. No injury occurred when high, carcinogenic doses were given orally. It has been speculated that TCE metabolites destroy P4502E1, the isoenzyme primarily responsible for its metabolic activation. They found instead that high doses of TCE induce P4502E1, apparently by binding to it and delaying its normal degradation (Lee *et al.*, 2000b). Low doses of TCE had no such effect. TCE is more efficiently eliminated by the liver and is expected to be less carcinogenic when the VOC is ingested over a prolonged time, than when it is ingested as a single dose.

CONCLUSIONS

Issues associated with the potential carcinogenicity of TCE and its metabolites have been debated for the past several decades. Health risk assessments of VOCs usually require

extrapolation of the results of toxicity studies in animals to predict toxic effects in humans. Physiologically based pharmacokinetic (PBPK) modeling is a powerful tool for rat-to-human extrapolation of the capacity of first-pass elimination of TCE with environmentally-relevant exposures. Highly sensitive, selective and accurate bioanalytical methods are necessary to obtain reliable animal data. SPME, a novel technique, is used in the study described in this dissertation. The optimization of headspace SPME conditions for the determination of TCE in rat plasma is presented in Chapter 2. The development and validation of a headspace SPME GC/MS method for the determination of TCE in rat blood and tissues is described in Chapter 3. Due to the extremely low levels of TCE in rats following environmentally-relevant exposures, a highly sensitive method using headspace SPME GC-NCIMS for the determination of TCE in biological samples is presented in Chapter 4. The LOQ for this NCI method is 10 times lower than the method described in Chapter 3. TCE is a lipophilic compound that prefers to accumulate in adipose tissue. Chapter 5 presents two sensitive methods for the determination of TCE in rat adipose tissue using SPME GC-EIMS and GC-NCIMS. In Chapter 6, an *in situ* derivatization/SPME GC-NCIMS method was validated for the determination of TCE metabolites including TCA, DCA and TCOH, in rat blood. All of the methods presented in these chapters were validated for specificity, linearity, accuracy and precision. The results were acceptable according to the current FDA bioanalytical validation requirements. By characterizing the toxicoketics and presystemic elimination of TCE in rats following environmentally-relevant exposures in Chapter 7 using the methods developed in the previous chapters, uncertainty in extrapolation of TCE toxicity data from high to low loses is reduced. This will improve the accuracy of assessment of human health risks from low-level TCE exposure.

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CHAPTER 2

OPTIMIZATION, VALIDATION AND APPLICATION OF A METHOD FOR DETERMINATION OF TRICHLOROETHYLENE (TCE) IN RAT PLASMA BY HEADSPACE SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY MASS SPECTROMETRY

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ABSTRACT:

Trichloroethylene (TCE) is a small halogenated compound that has been used extensively as a metal degreaser and a solvent for the past 100 years. As a result of its widespread use, TCE can be found in the groundwater of about one third of the hazardous waste sites on the United States Environmental Protection Agency's (EPA) National Priorities List. Human exposure to TCE in the environmental media is of concern because TCE has been found to be carcinogenic in laboratory animals. This paper describes the development and validation of a HS-SPME-GC/MS method for determination of TCE in rat plasma. Effects of different parameters such as sample volume, extraction and desorption conditions, fiber positions and salt addition were investigated and optimized. The method is rapid, simple, sensitive and requires a very small sample volume. The lower limit of quantitation is 0.25 ng/ml and correlation coefficient (R^2) values for the linear range of 0.25-100 ng/ml were 0.996 or greater. The precision and accuracy for intra-day and inter-day were better than 8.0%. This validated method was successfully applied to study the toxicokinetic behavior of TCE following low levels of oral administration.

1. INTRODUCTION

Trichloroethylene is an industrial solvent used primarily in metal cleaning and degreasing operations. It is also used as an intermediate for synthesis of other chemicals (e.g., polyvinyl chloride) and in varnishes, adhesives, paints, and lacquers (Bakke *et al.*, 2007). TCE also has many applications in a variety of household products such as spot removers, carpet cleaning fluids, typewriter correction fluids, and paint removers (Bakke *et al.*, 2007; ATSDR, 1997). Much of the TCE used in the United States is released into the atmosphere from vapor degreasing operations. TCE contamination of ground and surface waters is largely attributed to industrial discharge of the chemical and leaching from hazardous waste sites (ATSDR, 1997; Wu and Schaum, 2000). As a result of its widespread use, TCE can be found in the groundwater in the proximity of more than 50% of the hazardous waste sites on the United States Environmental Protection Agency's (EPA) National Priorities List (Fay and Mumtaz, 1996; Fay, 2007). Trichloroethylene is one of the most frequently reported organic contaminants in groundwater (Moran *et al.*, 2007; Fay and Mumtaz, 1996). According to the Third National Health and Nutrition Examination (NHANES III), an estimated 10% of the US population has detectable levels of TCE in their blood (Ashley *et al.*, 1994; Churchill *et al.*, 2001). Human exposure to TCE in the environmental media is of concern, because high doses of TCE have been found to be carcinogenic in laboratory animals (Bull, 2000; Rhomberg, 2000; EPA, 2001). Inhalation or ingestion of high doses of TCE for even a short time can produce central nervous system (CNS) depression, ranging from slight dizziness or sleepiness to unconsciousness and even death (ATSDR, 1997). Exposure to relatively high levels of trichloroethylene in air or water for a long period of time (years) can damage the liver and kidney (Green *et al.*, 1998). Increased incidences of renal cell carcinoma have been reported in workers exposed occupationally, for years to high

vapor levels of TCE (Lipworth *et al.*, 2006; Scott and Chiu, 2006; Raaschou-Nielsen *et al.*, 2003; Zhao *et al.*, 2005). Based on the limited epidemiology data, TCE was classified by the International Agency for Research on Cancer (IARC) and the National Toxicology Program (NTP) as a "probable human carcinogen" (National Toxicology Program, 2001; IARC, 1995). However, concentrations of TCE in drinking water supplies are usually very low, ranging from 0.02 ug/L to 230 ug/L (Moran *et al.*, 2007). The key question is whether environmentally encountered levels of TCE pose a cancer risk (NRC, 2006). In order to better evaluate the possible role of trace amounts of TCE in carcinogenesis, a reliable analytical method with high sensitivity for quantitation of TCE in biological matrices is needed.

Methods that have been published to detect TCE include purge and trap, headspace, and liquid-liquid extraction (Ashley *et al.*, 1994; Zoccolillo *et al.*, 2005; Muralidhara and Bruckner, 1999; Brown, Muralidhara *et al.*, 2003; Brown, Dixon *et al.*, 2003; Delinsky *et al.*, 2005). Ashley et al. report a GC-MS method using purge and trap for the determination of parts per trillion levels of 32 volatile organic compounds (VOCs), including TCE, in 10 mL of human blood (Ashley *et al.*, 1994). Their method is sensitive, but obtaining 10 mL of blood from laboratory animals for toxicokinetic and mechanistic studies is not realistic. Mice and rats are used for most such experiments. Zoccolillo et al. described an improved assay of TCE in water by purge and trap with GC-MS (Zoccolillo *et al.*, 2005). Their LOQ is 1 ng/L, but 10 mL of water is required to achieve this sensitivity. Purge and trap analysis is time consuming, and requires a special apparatus to maintain low temperatures (-100 ºC). Muralidhara and Bruckner report a headspace GC-ECD method with a LOQ of 50 ng/ml for the measurement of TCE and its metabolites in blood (Muralidhara and Bruckner, 1999). Traditional headspace GC analysis is not sensitive enough, because this technique does not involve sample pre-concentration. Another technique

involves liquid-liquid extraction (LLE) with ether followed by GC-MS analysis of TCE (Brown, Muralidhara *et al.*, 2003; Brown, Dixon *et al.*, 2003). However, it has been shown that many solvents used in LLE are contaminated with TCE (Brown, Dixon *et al.*, 2003). These methods require additional sample preparation and often extensive solvent purification steps. This results in increased cost, time, potential error and decreased sensitivity (LOQ=5 ng/ml) in the analysis.

Solid phase microextraction (SPME) can pre-concentrate the samples, minimize the use of extraction solvents, and minimize sample preparation time. With this method, the coated fiber is exposed to the sample or its headspace and the target analytes partition from the sample matrix to the coating. The fiber bearing the concentrated analyte is then transferred to the analytical instrument where desorption, separation, and quantification of the extracted analyte takes place (Alpendurada, 2000). Several methods have been published describing the use of SPME for analysis of TCE. Some of them are focused on the measurement of TCE in environmental media, such as air, drinking water and soil (Pastore *et al.*, 2005; Larroque *et al.*, 2006; Bocchini *et al.*, 1999). Quantitation of chemicals in a biological matrix is difficult due to matrix effects. Dehon et al. report a SPME GC-MS method for determination of TCE in tissues (Dehon *et al.*, 2000). Relatively large volumes of samples are required for their method. Optimization and validation of the method are not described in this paper. Poli developed a sensitive method with a detection limit of 0.01 μg/L for measurement of TCE in human urine using headspace SPME GC-MS (Poli *et al.*, 2005). Two ml of urine sample was used in their method. This large a sample volume cannot be obtained from small animals where multiple sampling is necessary for toxicokinetics studies. We previously developed a SPME procedure for analysis of TCE in rat tissues with a LOQ of 5 ng/mL (Dixon *et al.*, 2005). The method was not validated and its sensitivity not adequate for use in toxicokinetic studies of environmentally relevant levels of TCE. The ability

to monitor the time-course of TCE in rodents given very low doses of the chemical would be quite valuable to toxicologists and cancer risk assessors. There are no toxicokinetic data for extremely low-level TCE exposures. The present paper describes the optimization and validation of a SPME GC-MS method for the determination of TCE in plasma. This method is very sensitive and utilizes small sample volumes obtained from rodents. Its lower limit of quantitation (LLOQ) of TCE is 0.25 ng/mL.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Analytical grade trichloroethylene (TCE) and ammonium sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, sodium fluoride, sodium carbonate, and sodium sulfate were of analytical grade purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water used in experiments was generated from a Continental deionized water system (Natick, MA, USA). Ultra high purity (UHP) helium was purchased from National Welders (Charlotte, NC, USA). Solid phase microextraction fibers coated with 100 μm polydimethylsiloxane were purchased from Supelco (Bellefonte, PA, USA). All fibers were conditioned at 250 ºC for 30 min prior to use. One fiber can be used for more than one hundred biological samples. Alkamuls, the emulsifying agent used in preparing an aqueous emulsion of TCE for the animal study, was obtained from Rhone-Poulenc (Cranbury, NJ, USA).

2.2 Gas chromatography-mass spectrometry

The analyses were carried out on an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 mass selective detector (Palo Alto, CA, USA). Separation of analytes was obtained on a ZB-5 column (Phenomenex, Torrance, CA, USA: 30m×0.25mm i.d., 0.25 μm

thickness) using helium as the carrier gas (flow rate, 1 ml/min). The GC injection port temperature was maintained at 200 ºC in the splitless mode. The oven conditions were: 35 ºC hold for 3 min, increase of 10 ºC /min to 100 ºC, hold for 2 min, aux transfer line temperature, 280 ºC. The mass spectrometer was operated in positive electron ionization (EI) mode with an electron energy of 70 eV. Quantitation of TCE was performed using the selected-ion monitoring (SIM) mode by monitoring m/z 130 (quantitation ion), m/z 132 and m/z 134 (confirmation ions). A solvent delay of 1.5 min was set to protect the filament from oxidation.

2.3 Preparation of working standard and quality control solutions

A stock solution of TCE was prepared in acetonitrile to yield a final concentration of 100 mg/ml. Standard solutions for the calibration curve were prepared from the stock solution in the following concentrations: 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng/ml. Standards used to assess precision and accuracy were prepared in acetonitrile from the stock solution in concentrations of 2.5, 7.5, 75 and 750 ng/ml. All stock and standard solutions were refrigerated at 4 ºC during the day of use and were prepared fresh daily.

2.4 Sample preparation and headspace SPME procedure

Two hundred µl plasma samples spiked with TCE were transferred to 2.0 ml SPME vials containing 100 µl of an ammonium sulfate solution (30%) and quickly sealed with a PTFE-coated silicone septum and an aluminum cap. Headspace sampling was performed using a 100 µm PDMS fiber (Sulpelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland). Sample vials were preheated in an agitator for 5 min before analysis. The SPME fiber was then exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 15 min at 30 ºC under agitation, the fiber

was withdrawn into the needle and immediately desorbed at 200 ºC for 2 min into the GC injection port.

2.5 Method validation

The method was validated for linearity, recovery, accuracy and precision. For the determination of the assay linearity, blank rat plasma was spiked with 20 µl of TCE standard solutions to yield TCE plasma concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 ng/ml. Calibration curves were generated by linear regression analyses of the peak area of TCE against the concentration applying a weighting factor ($1/x^2$). Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples (0.25, 0.75, 7.5 and 75.0 ng/ml). Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Relative recoveries were calculated for spiked samples at 0.25, 0.75, 7.5 and 75 ng/ml (n=5) by dividing the peak area for TCE spiked in rat plasma by the peak area for an equal concentration of TCE in deionized water. As TCE is volatile, every precaution was taken to ensure it was not lost during analysis. The autosampler stability was evaluated over a period of 12 hours to determine if there was any loss of signal due to the time a sample spends in the autosampler prior to analysis. Freeze/thaw stability was investigated by comparing the stability of samples following three freeze/thaw cycles against freshly spiked samples. The area response for each cycle was compared and the %RSD calculated. Stability testing was performed at concentrations of 7.5 and 75 ng/ml.

2.6 Sampling

Male Sprague-Dawley (S-D) rats were obtained from Charles River Laboratories (Raleigh, NC, USA). The rats were acclimated for at least 7 days in an AALAC-approved animal care facility after arrival. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia. Rats were cannulated so that serial blood samples could be taken to characterize the time-course of TCE. Briefly, each rat was anesthetized and a cannula surgically inserted into the right carotid artery. Water was provided *ad libitum*, but food was withheld during the 24-h recovery period before dosing. Food was provided 3 h after dosing. TCE was given orally by gavage in a dose of 1 mg/kg using Alkamulus as the vehicle. Blood samples from each rat (n=3) were taken 10, 30, 60, 120, and 240 minutes post dosing. Plasma was obtained by centrifuging whole blood at 2500× g at 4 ºC for 10 minutes.

3. RESULTS AND DISCUSSION

3.1 Optimization of the HS-SPME conditions

For the measurement of TCE in plasma, headspace (HS)-SPME was preferred to direct sampling for several reasons: equilibrium times are generally shorter in the gas phase than liquid phase (Lord and Pawliszyn, 2000; Louch *et al.*, 1992); avoiding direct contact of the fiber with the sample protects the fiber; reduction of matrix effects; and increased method sensitivity (Lord and Pawliszyn, 2000). In HS-SPME, rules based on the equilibrium between three phases (aqueous phase, coating phase and headspace) are employed. The equilibrium conditions can be described as (Zhang and Pawliszyn, 1993):

$$n_f = \frac{K_{fw} V_f n_0}{K_{fw} V_f + K_{hw} V_h + V_w}$$

Where n_f is the number of molecules on the fiber, n_0 is the initial number of molecules in the aqueous phase, K_{fw} is the fiber coating/aqueous distribution constant, K_{hw} is the headspace/aqueous distribution constant, V_f is the volume of the coating, V_h is the headspace volume, and V_w is the volume of the aqueous phase. The equilibrium constant values in Eq. (1) are affected by temperature, addition of salt, sample pH and in the presence of organic solvents. SPME methods often involve a number of stages. In order to optimize HS-SPME sampling conditions in the current study, we evaluated the possible effect of several variables known to affect the extraction efficiency. These included sample volume, desorption time, desorption temperature, fiber position in the sample vial and GC-injector, sample preheating time, extraction time and temperature, and amount and type of salt added to samples. A commercially available 100-μm PDMS fiber was chose for this study, based on its high affinity for volatile organic compounds (VOCs), such as TCE. For method optimization, peak areas of TCE were compared at medium levels of 25 ng/ml.

3.1.1 Sample volume

Due to the need for serial sampling in toxicokinetic studies with rodents, a small volume of plasma is required. Repetitive 200-μl plasma samples can easily be taken from rats. Use of 10-ml vials dramatically increases the volume of headspace. The analytes are diluted in the headspace before they are extracted by the fiber. Considering Eq. (1), 2-ml vials were selected. When using 200 μl of plasma, the method sensitivity with 2-ml vials was almost 5 times higher than with 10-ml vials.

3.1.2 Temperature effect and equilibrium time

SPME fiber equilibration is an exothermic process. Any increase in sampling temperature will decrease both analyte recovery and equilibrium extraction time (Zhang and

Pawliszyn, 1993; Pawliszyn, 1997). The headspace/sample partition coefficient of the analyte increases with increase in temperature, but the fiber coating/headspace partition coefficient decreases. The first phenomenon leads to a higher concentration of the analyte in the headspace, and the latter results in a lower amount of analyte the fiber is able to extract at equilibrium. The effects of extraction temperature and time on peak areas of TCE are shown in Fig. 2.1. Comparison of the extraction time profiles obtained at different temperatures revealed that a higher sampling temperature increased the speed of equilibrium, but decreased the amount of extracted analyte on the fiber. At 30 and 40 ºC, sample response increased with the increase in extraction time. The equilibrium status can be reached within 15 min. At 50 and 60 ºC, the equilibrium extraction was achieved in 5 min, but the extracted amounts of TCE decreased dramatically due to a lower fiber coating/headspace partition coefficient. Therefore, an extraction time of 15 min and a temperature of 30 ºC were selected as the optimum extraction conditions.

3.1.3 Sample preheating time

At a low extraction temperature, samples need to be preheated for some time before the fiber is exposed to the headspace for extraction. This preheating process will improve the mass transfer of analyte from the liquid sample to the headspace. Fig. 2.2 shows the effect of the preheating time on the peak area of TCE. The preheating time was varied from 0-20 minutes. From 0-5 minutes, the TCE peak area increased with increased preheating time. After 5 minutes, there did not appear to be significant difference in sample response. As a result, 5 minutes was chosen as the preheating time.

3.1.4 Fiber position

For HS-SPME, the depth of the fiber in the vial should be adjusted, so it is above the meniscus of the liquid phase. By varing the penetration depth of the fiber into the autosampler

vials from 22-31 mm (Fig. 2.3), it was found that there was no significant effect on TCE peak area. Twenty-five mm was selected as the fiber length. The position of the fiber in the GC injector needs to be optimized as well, because the temperature in the GC injector is not distributed evenly. Usually the fiber should be desorbed in the middle of the liner. The optimization results (Fig. 2.4) illustrate that 45 mm is the optimum length of the fiber exposed in the GC injector, although there is no significant effect on TCE response over this range of lengths.

3.1.5 Salt effect

Salt can increase or decrease the amount of analyte extracted. Therefore, the influence of salt addition on the efficiency of SPME was studied. The presence of salt increases the ionic strength of solutions and can affect the solubility of analytes in biological samples. Solid salts were initially used in the experiments. Addition of salts as solids had no significant effect on sensitivity. A possible reason for this phenomenon was that the addition of solid salts increased the viscosity of the solution, which resulted in lower mass transfer of TCE from the plasma to the headspace. Thus, salt solutions were used in the experiments. The effects of five types of salt solutions were studied (Fig. 2.5). Sodium fluoride, sodium chloride, sodium sulfate, sodium carbonate and ammonium sulfate were dissolved in deionized water to prepare different concentrations of salt solutions. For each plasma sample, 100 ul of each salt solution was added. The experiment revealed that a different concentration of each salt was required to maximize the method's sensitivity (i.e. 10% for NaCl, 30% for $(NH_4)_2SO_4$, 2% for NaF, 15% for Na_2SO_4 and 2% for Na_2CO_3). When the salt concentration was above the optimum value, a decrease in response was observed, because excess salt decreased TCE's volatility and thus the extraction efficiency. Use of NaCl resulted in the lowest signal. Both $(NH_4)_2SO_4$ and NaF produced high

responses due to their protein denaturing properties. A solution containing 30% ammonium sulfate was selected, due to the salt's high solubility and strong ionic strength.

3.1.6 Desorption temperature and time

It is quite important to keep the time interval required for desorption as short as possible to avoid carryover effects. Therefore, the highest temperature that can be used without damaging the fiber should be applied. At high GC injector temperatures, the partition coefficient between the fiber coating and the headspace is decreased, so that desorption of the analyte takes place. Fig. 2.6 shows the effect of desorption time on extraction efficiency of TCE. The desoprtion of extracted TCE on the fiber increased with increased desorption time. After 2 minutes, all of the TCE was desorbed and no carryover effects were observed. Therefore, a 2-minute period was chosen as the optimal desorption time. The influence of desorption temperature on TCE peak area was examined over the range of 100-270 ºC (Fig. 2.7). The optimum temperature was found to be 200 ºC. When the injection port was maintained at 150 ºC or less, a split or tailing chromatographic peak was observed. This likely occurred because the TCE did not desorb from the fiber quickly. Higher temperatures such as 250 or 270 ºC did not increase the sensitivity, because desorption at 200 ºC for 2 minutes was sufficient to transfer all of the analyte from the fiber into the GC injector. In addition, at higher temperatures, more coating from the fiber will be lost, resulting in shortened fiber life time. Thus, a desorption temperature of 200 ºC for 2 min was selected.

3.1.7 Effect of organic solvent on extraction efficiency

At 25 ºC, the maximum solubility of TCE in water is 1.366 g/L. Due to its low solubility, extraction of TCE from water is inefficient. Organic solvent can increase the solubility of TCE and decrease the binding of TCE to proteins. Different volumes of acetonitrile and methanol

were added as modifiers to plasma samples spiked with TCE dissolved in water. The TCE peak areas with an organic solvent were found to be 3-4 times larger. Due to the protein precipitation properties of acetonitrile, it was chosen as the solvent for preparation of TCE solutions.

3.2 Validation of the method

Representative chromatograms obtained from blank plasma and plasma spiked with the LLOQ standard (0.25 ng/ml) are shown in Fig. 2.8. No interfering peaks from endogenous compounds were observed at the retention times of TCE. Utilization of selected-ion monitoring (SIM) mode enhanced the mass spectrometric selectivity by eliminating the need to scan a large range of masses.

The calibration curves for each day of validation and analysis of plasma showed a linear response (R^2>0.996) over the range from 0.25 to 100 ng/ml. SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used each day of the validation and analysis. The LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 0.25 ng/ml for TCE, as shown in Table 2.1. Assay precision and accuracy for TCE were calculated over a range of 3 days. Table 2.1 summarizes the accuracy and precision data that were collected. Both the intra-day precision (n=5) and inter-day precision (n=15) were lower than 7.81% (%R.S.D.); both the intra-day accuracy (n=5) and inter-day accuracy (n=15) were lower than 7.03% (%error).

While recovery studies are normally performed for bioanalytical method validation, the values are not normally reported for SPME studies. However, relative recovery is a good parameter for evaluating the matrix effect of a method. The recovery of TCE from plasma was

examined versus water. Relative recoveries of TCE in rat plasma at concentrations of 0.25, 0.75, 7.5 and 75 ng/ml (n=5) were 66.64, 66.74, 72.19 and 65.71%, respectively.

Assessing the stability of analytes during analysis is very important. The total HS-SPME sampling time for TCE was about 22 min. Therefore, when accumulating multiple plasma samples for analysis, the samples may sit in the autosampler for hours. The stability of plasma samples remaining in the autosampler for 12 hours was examined. No significant decrease in sensitivity was observed. Another type of stability test is freeze-thaw stability. It is often necessary to freeze samples prior to analysis. Therefore, it is important to demonstrate that samples that have been frozen and then thawed provide equivalent responses to those that have not been frozen. The freeze/thaw stability tests indicated the TCE was stable for three freeze/thaw cycles. The %R.S.D. for three cycles was less than 10%.

3.3 Applications

The validated method was applied to quantify TCE in rat plasma following oral administration of a low dose at 1 mg TCE /kg of body weight. Fig. 2.9 shows the resulting TCE concentration versus time profile. The first time-point for sample collection was 10 min. However, the T_{max} for TCE following oral administration is usually 4-5 min (D'Souza *et al.*, 1985; Lee *et al.*, 1996). Thus, there is no TCE absorption phase in this profile. These toxicokinetic data reveal that low doses on TCE are very rapidly cleared from the blood stream of male rats. Rapid metabolism and exhalation are responsible for TCE elimination from the systemic circulation. After 4 hours, most all of the TCE was eliminated from the body, and the plasma concentrations approached the limit of quantitation.

4. CONCLUSIONS

A simple, specific, rapid and sensitive SPME-GC/MS method for the determination of TCE in plasma has been developed and validated. Several influential parameters including sample volume, extraction and desorption conditions, fiber positions and salt were characterized and optimized. To our knowledge, this is the first optimized and validated SPME-GC/MS method for determination of TCE in plasma. The technique yielded excellent linearity, precision and accuracy over a wide calibration range. The limit of quantition was 0.25 ng/ml, which is 20-fold lower than that of reported liquid-liquid extraction GC/MS methods and 100-fold lower than the headspace GC-ECD methods (Muralidhara and Bruckner, 1999; Brown, Muralidhara *et al.*, 2003). This method was successfully applied to study the toxicokinetic of TCE in arterial plasma following ingestion of a very small amount of TCE.

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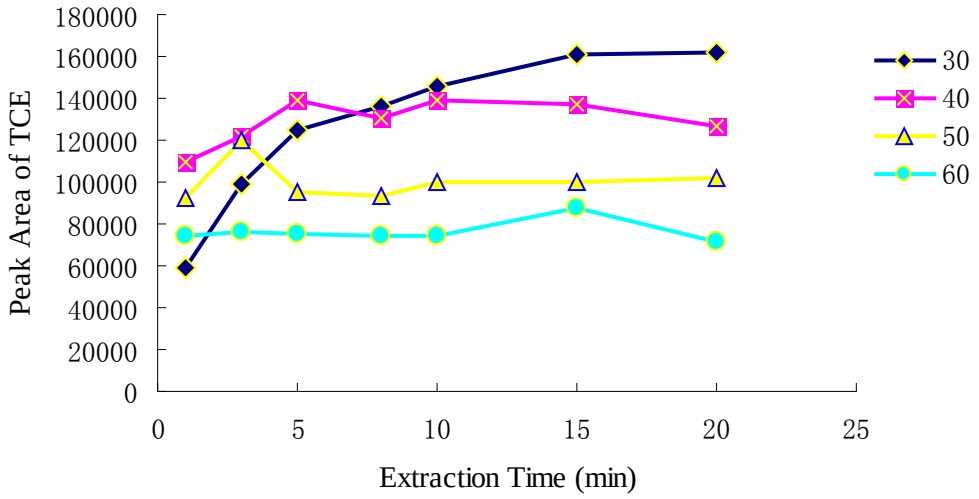

Figure 2.1. Effects of temperature and time on extraction efficiency

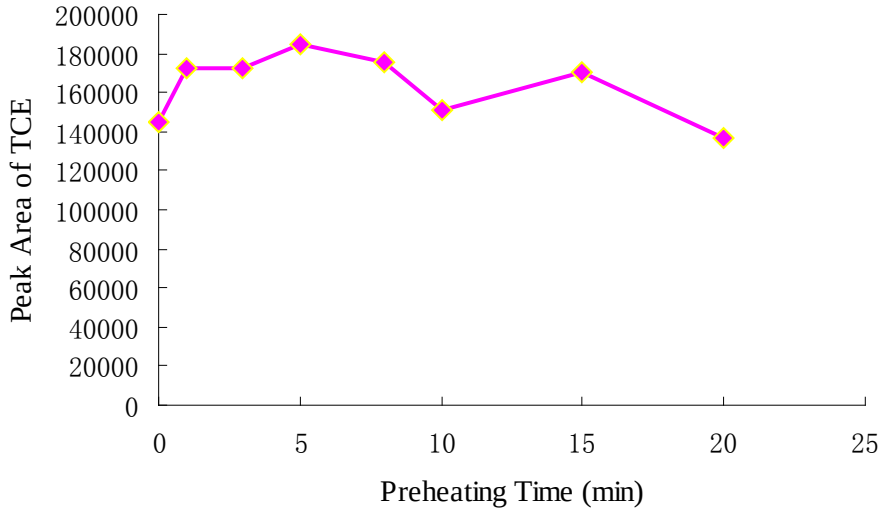

Figure 2.2. Effect of sample preheating time on SPME efficiency

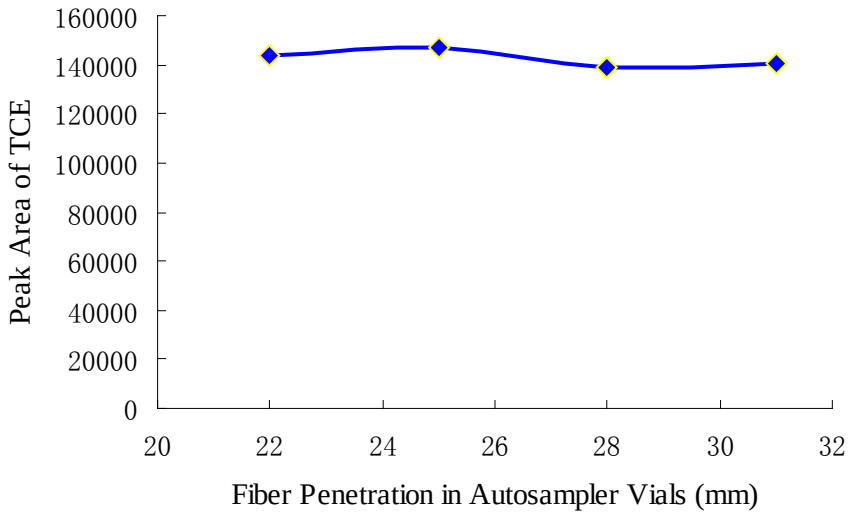

Figure 2.3. Effect of SPME fiber penetration in autosampler vials on efficiency

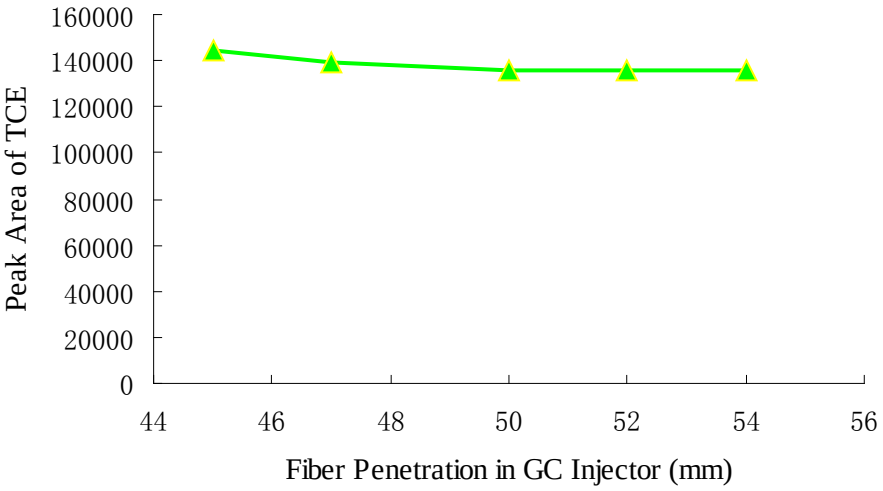

Figure 2.4. Effect of SPME fiber penetration in GC injector on efficiency

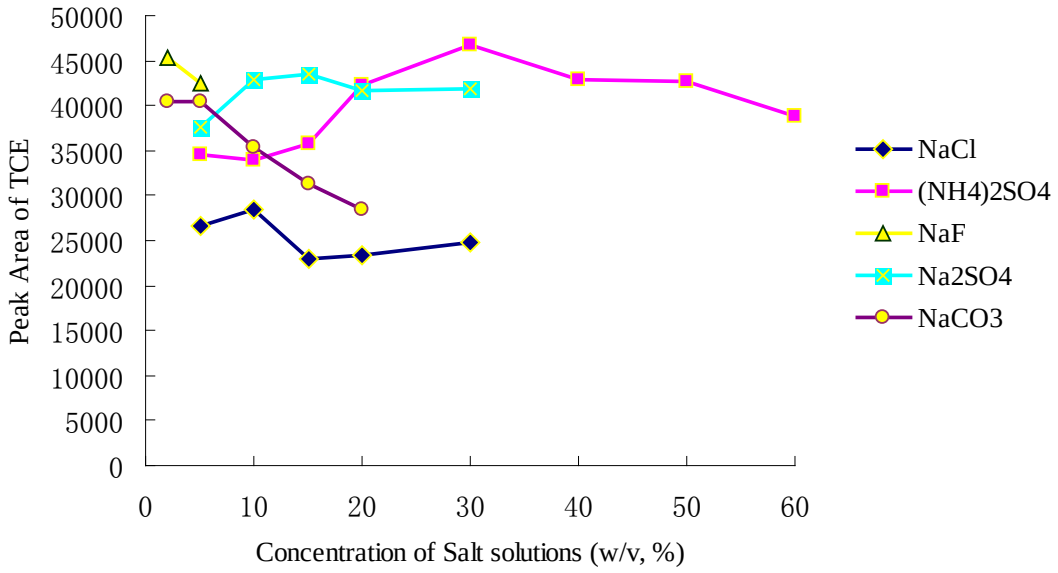

Figure 2.5. Effects of salt addition on SPME extraction efficiency

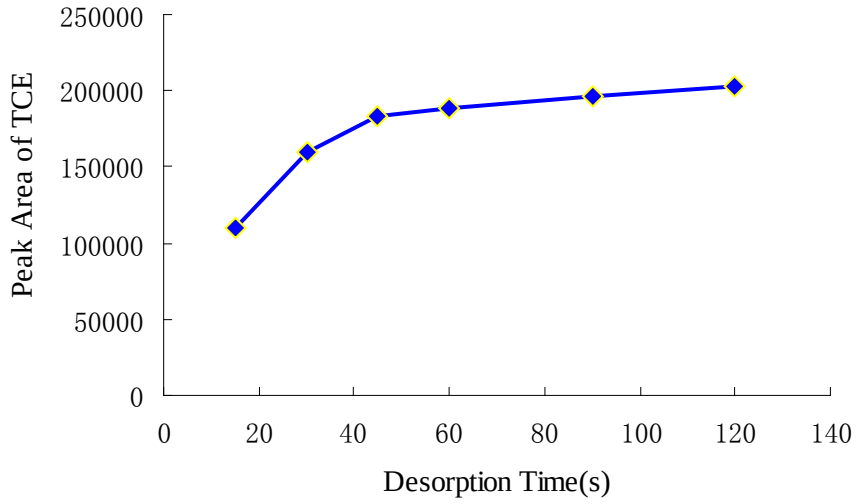

Figure 2.6. Effect of SPME fiber desorption time on TCE extraction efficiency

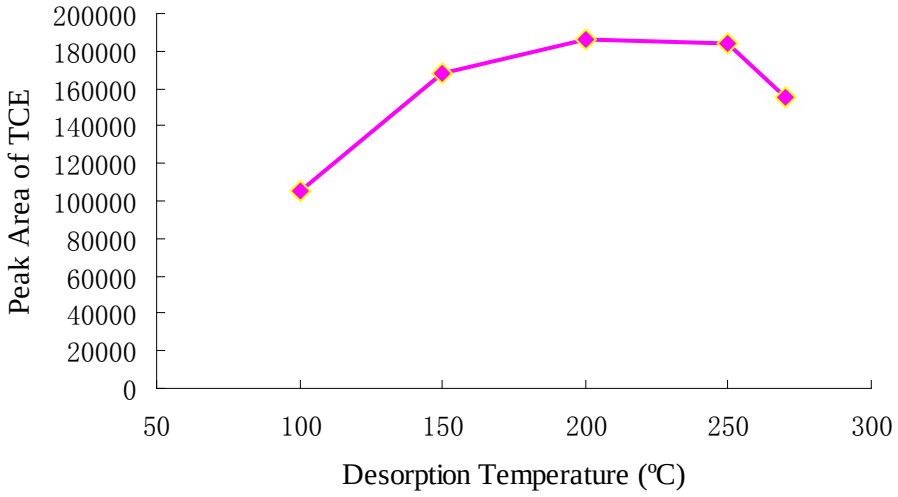

Figure 2.7. Effect of SPME fiber desorption temperature on efficiency.

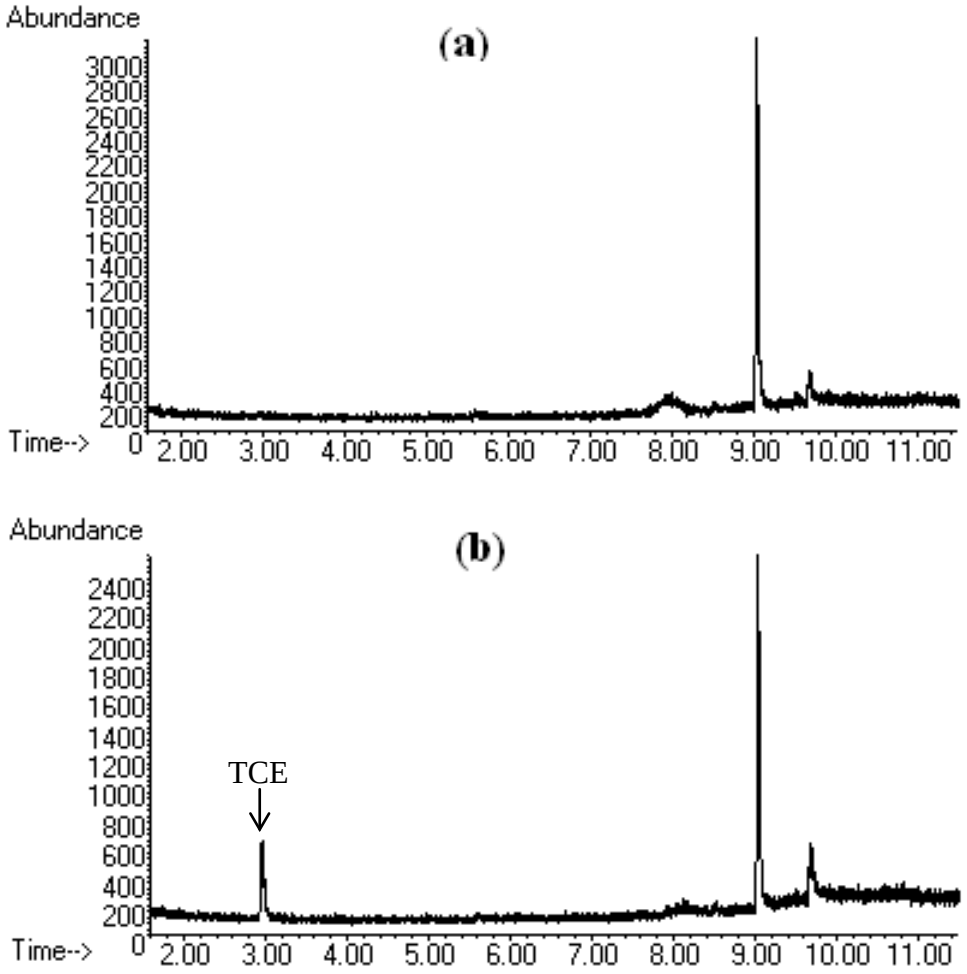

Figure 2.8. Representative chromatograms obtained from (a) blank rat plasma; (b) plasma spiked with LLOQ (0.25 ng/ml) concentration of TCE.

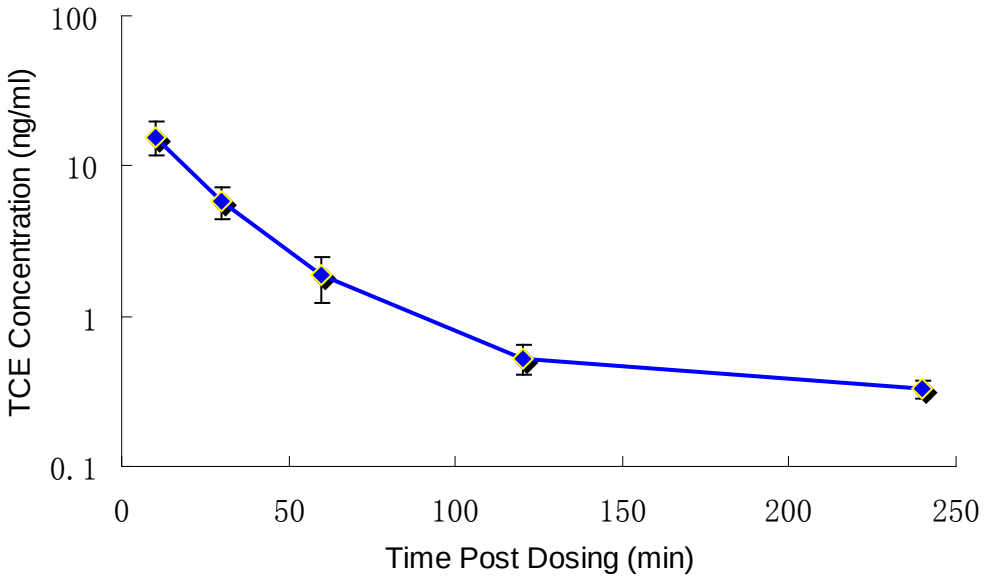

Figure 2.9. Time versus plasma TCE concentration profile in S-D rats administered a single oral dose of 1 mg/kg. (n=3)

Table 2.1. The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the SPME-GC/MS method used to quantitate TCE in rat plasma

	Intra-day			Inter-day		
Concentration TCE added (ng/ml)	Concentration TCE found (ng/ml)	R.S.D. (%)	Error (%)	Concentration TCE found (ng/ml)	R.S.D. (%)	Error (%)
0.25	0.256±0.020	7.68	7.03	0.252±0.020	7.81	6.80
0.75	0.699±0.023	3.32	6.80	0.724±0.032	4.36	4.46
7.5	7.004±0.256	3.65	6.61	7.000±0.313	4.48	6.70
75	74.32±4.277	5.76	4.28	75.76±2.820	3.82	3.27

CHAPTER 3

DETERMINATION OF TRICHLOROETHYLENE IN BIOLOGICAL SAMPLES BY HEADSPACE SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY MASS SPECTROMETRY

Liu Y, Muralidhara S, Bruckner JV, Bartlett MG. *Journal of Chromatograpgy B.* 2008; **863**(1): 26-35. Reprinted here with the permission of publisher (Copyright Elsevier)

ABSTRACT

A simple, rapid and sensitive method for determination of trichloroethylene (TCE) in rat blood, liver, lung, kidney and brain, using headspace solid-phase microextraction (HS-SPME) and gas chromatography/mass spectrometry (GC/MS), is presented. A 100-μm polydimethylsiloxane (PDMS) fiber was selected for sampling. The major analytical parameters including extraction and desorption temperature, extraction and desorption time, salt addition, and sample preheating time were optimized for each of the biological matrices to enhance the extraction efficiency and sensitivity of the method. The lower limits of quantitation for TCE in blood and tissues were 0.25 ng/ml and 0.75 ng/g, respectively. The method showed good linearity over the range of 0.25-100 ng TCE/ml in blood and 0.75-300 ng TCE/g in tissues, with correlation coefficient (R^2) values higher than 0.994. The precision and accuracy for intra-day and inter-day measurements were less than 10%. The relative recoveries of TCE respect to deionized water from all matrices were greater than 55%. Stability tests including autosampler temperature and freeze and thaw of specimens were also investigated. This validated method was successfully applied to study the toxicokinetics of TCE following administration of a low oral dose.

1. INTRODUCTION

Trichloroethylene (TCE) is a halogenated volatile organic compound (VOC) that has been used extensively as a metal degreaser, chemical intermediate, anesthetic and dry cleaning agent. The presence of TCE in the environment can be attributed to industrial discharge of the chemical to water and land and leaching from hazardous waste sites [1-3]. As a result of its widespread use and migration through soil, TCE can be found in the groundwater at more than 50% of the hazardous waste sites on the United States Environmental Protection Agency's (EPA) National Priorities List [4, 5]. According to a survey by the National Water-Quality Assessment (NAWQA) program, 5% of wells throughout the United States have detectable levels of TCE, with concentrations ranging from 0.02 ug/L to 230 ug/L [6]. Exposure to high doses of TCE can result in central nervous system depression, liver changes and cardiac arrhythmias[2]. Exposure of the general population to very low levels of TCE in environmental media is of concern primarily because of potential carcinogenic risk [7-10].

The human body possesses a number of defense mechanisms to protect against low-level toxic and mutagenic insults. These mechanisms include presystemic elimination, metabolic detoxification, DNA repair, death of mutated cells, apoptosis, destruction of mutated cells by the immune system, and the action of tumor suppressor genes. The extremely high doses of TCE and other VOCs administered in rodent cancer studies "overwhelm" these protective processes, kill cells, exceed the capacity of tissue repair, and can then cause tumors in some strains of mice and rats.

A substantial amount of TCE and other VOCs may be removed from the portal venous blood by presystemic or first-pass elimination. Drugs and chemicals absorbed from the gastrointestinal tract must first pass though the portal blood into the liver and on to the lungs,

before entering the arterial circulation and being transported to tissues throughout the body [11, 12]. It has been theorized but not demonstrated experimentally, that low oral doses of VOCs are completely removed by presystemic elimination [13, 14]. Lee et al. characterized the presystemic elimination of TCE in rats [15]. These experimental results demonstrated that the liver and lungs can remove a significant proportion of moderate oral doses of TCE. Lack of analytical sensitivity, however, precluded working with more environmentally-relevant levels. In order to directly measure the capacity of first-pass elimination of TCE under environmental exposure conditions, a valid, sensitive and rapid analytical method is needed to measure trace concentrations of TCE in multiple blood and tissues samples**.**

Methods that have been published to quantify TCE in biological samples include purge and trap, headspace, and liquid-liquid extraction [16-21]. Methods using purge and trap are extremely sensitive, but require large volume samples. The LOQ is usually in pg/ml levels [16, 17]. Large volumes of blood (e.g., 10 ml) are not realistic for toxicokinetic studies in small rodents. Traditional headspace methods are less sensitive than purge and trap procedures because they do not involve sample pre-concentration. A LOQ of 50 ng/ml was reported using headspace GC-ECD for the measurement of TCE and its metabolites in blood and tissues [18]. Other methods involving liquid-liquid extraction (LLE) with ether, followed by GC-MS analysis of TCE have been reported [19, 20]. However, it has been found that many solvents used in LLE are contaminated with TCE [20]. These methods also involve a number of additional sample preparation steps. This results in increased cost, time, potential error and decreased sensitivity (LOQ=5 ng/ml) in the analysis.

Solid phase microextraction (SPME) is an innovative, solvent-free technology that is fast, economical, and versatile. With this method, a coated fiber is exposed to the volatilized

chemical in the sample's headspace. The analyte partitions from the sample matrix to the coating. The fiber bearing the concentrated analyte is then transferred to the analytical instrument where desorption, separation, and quantification of the extracted analyte takes place [22]. SPME has several advantages over classical GC techniques. SPME can pre-concentrate the samples due to the high affinity of the analyte for the fiber coating. Potentially contaminated organic solvents commonly used for extracting volatile analytes from biological sample are not needed. Therefore with SPME, sample handling is minimized. When a biological sample is taken, it is simply placed into an autosampler vial and capped until analysis. This limited sample preparation helps to minimize the loss of volatile analytes and reduces potential error. Furthermore, headspace SPME methods can reduce matrix effects, because the biological macromolecules in the sample do not volatilize into the headspace. Several methods have been published describing the use of SPME for the analysis of TCE [23-30]. Some of these are focused on the determination of TCE in environmental media, such as air, drinking water and soil [23-26]. However, quantitation of chemicals in a biological matrix is much more difficult. Dehon et al. reported a SPME GC-MS method for determination of TCE in tissues [27]. Relatively large volume samples were used in their method. Optimization and validation of the method were not addressed in this paper. Poli developed a sensitive method for measurement of TCE in human urine using headspace SPME GC-MS with a detection limit of 0.01 μg/L [28]. But the sample volume used for their method was 2 mL. This large volume cannot be obtained in toxicokinetics studies with rodents and other small animals. Xu et al. reported a GC-MS method coupled with SPME for the determination of TCE in environmental biological samples, such as the microcosm of a cell [29]. Their method's detection limit was 5 μg/L, but complete immersion of the fiber in the biological samples substantially shortens the life of the fiber coating. In previous work in our laboratory, a SPME

method for analysis of TCE in rat tissues with a LOQ of 5 ng/mL was developed [30], but not validated. Its sensitivity was not sufficient to study the absorption, disposition and excretion of environmentally-relevant levels of the VOC.

Development and validation of physiologically-based pharmacokinetic (PBPK) models for TCE require blood and tissue concentration time-course data for the four primary target organs (i.e. liver, kidney, lung, and brain) [31-33]. We developed a HS-SPME GC-MS method for the determination of TCE in rats, in order to directly assess the extent of first-pass elimination of TCE in rats and to obtain data to develop and validate a PBPK model that can accurately forecast first-pass metabolism for any of a variety of exposure scenarios. This new method is quite sensitive and uses small sample sizes. The lower limit of quantitation (LLOQ) is 0.25 ng TCE/ml in blood and 0.75 ng/g in tissues.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Analytical grade trichloroethylene (TCE) and ammonium sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, sodium fluoride, sodium carbonate, sodium sulfate and sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water used in experiments was generated from a Continental deionized water system (Natick, MA, USA). Ultra high purity (UHP) helium was purchased from National Welders (Charlotte, NC, USA). Alkamuls, the emulsifying agent used in preparing doses for the animal study, was obtained from Rhone-Poulenc (Cranbury, NJ, USA).

2.2 Gas chromatography-mass spectrometry

The analyses were carried out on an Agilent 6890 gas chromatograph (GC) coupled with a model 5973 mass selective detector (Palo Alto, CA, USA). The GC was equipped with a 0.75 mm i.d. SPME liner. Separation of the analytes was obtained on a ZB-5MS column (Phenomenex, Torrance, CA, USA: 30m×0.25mm i.d., 0.25μm film thickness) using helium as a carrier gas (flow rate, 1 ml/min). The GC injection port and interface transfer line were maintained at 200 and 280 ºC, respectively. During the fiber desorption process, the splitless mode of injection was operated. After 2.5 min, the split vent valve opened to sweep any residual vapors from the liner. The oven temperature was initially held at 35 ºC for 3 min, then increased to 100 ºC at 10 ºC /min, and held for 2 min. The mass spectrometer was operated in positive electron ionization (EI) mode with an electron energy of 70 eV. Quantitation of TCE was performed using selected-ion monitoring (SIM) mode by monitoring m/z 130 (quantitation ion), m/z 132 and m/z 134 (confirmation ions). A solvent delay of 1.5 min was set to protect the filament from oxidation.

2.3 Preparation of working standard and quality control solutions

A stock solution of TCE was prepared in acetonitrile to yield a final concentration of 100 mg/ml. Standard solutions for the calibration curve were prepared from the stock solution in the following concentrations: 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng/ml. Standards used to assess precision and accuracy were prepared in acetonitrile from the stock solution in concentrations of 2.5, 7.5, 75 and 750 ng/ml. All stock and standard solutions were refrigerated at 4 ºC during the day of use and were prepared fresh daily.

2.4 Sample preparation

Prior to extraction, liver, kidney, lung and brain samples were homogenized with two volumes of deionized water (w/v) using a homogenizer (Polytron®, Brinkman, Switzerland). Samples for the calibration curves and quality control (QC) samples were prepared by adding 20 µl of the TCE standard into 200 µl of blank blood or blank tissue homogenate. This yielded calibration standard concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 ng/ml in blood and 0.75, 1.5, 3.0, 7.5, 15.0, 30.0, 75.0, 150.0 and 300.0 ng/g in tissues. The final concentrations of QCs were 0.25, 0.75, 7.5, 75.0 ng/ml in blood and 0.75, 2.25, 22.5, 225.0 ng/g in tissues. For blood samples, 200 µl of blood and 400 µl of sulfuric acid (1 mol/L) were added into a 2.0 ml SPME vial. For liver, kidney and lung tissues, 200 µl of tissue homogenate were transferred in 2-ml vials containing 200 µl of an ammonium sulfate solution (30%, w/v). Brain was treated in the same manner as liver, kidney and lung, but 200 µl of 5% NaCl solution (w/v) was used instead of 30% ammonium sulfate. All the autosampler vials were quickly sealed with PTFE-coated silicone septa and crimped aluminum caps. The vials were vortexed for 2 min and placed into the autosampler for analysis. Two blanks were run before each batch of samples to insure there was not a detectable background level of TCE before beginning.

2.5 Headspace SPME procedure

Headspace SPME sampling was performed using a 100-µm polydimethylsiloxane (PDMS) fiber (Sulpelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland). Fibers were conditioned at 250 ºC for 30 min prior to use. Sample vials were preheated in the agitator for 5 min before analysis, and the SPME fiber was then exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 5 min (blood and brain), 15 min (lung and kidney) or 20 min (liver) at 30 ºC

under agitation, the fiber was withdrawn into the needle and immediately desorbed at 200 ºC for 2 min into the GC injection port.

2.6 Method validation

The methods were validated for linearity, recovery, accuracy and precision. Calibration curves were generated by linear regression analyses of the peak area of TCE against the concentration applying a weight ($1/x^2$). Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four QC samples. Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Relative recoveries from the blood method were calculated for spiked samples at 0.25, 0.75, 7.5 and 75 ng/ml (n=5) by dividing the peak area for TCE by peak area for an equal concentration of TCE in deionized water. Relative recoveries from tissues were calculated for spiked samples at 0.75, 2.25, 22.5 and 225.0 ng/g (n=5) in the same manner. Because TCE is volatile, every precaution was taken to ensure it was not lost during analysis. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls [34]. The autosampler stability was evaluated over a period of 24 hours to determine if there was any loss of signal due to the time a sample spends in the autosampler prior to analysis. The freeze/thaw stability was investigated by comparing the stability of samples following three freeze/thaw cycles against freshly spiked samples. The stability testing was performed at 7.5 ng/ml in blood samples and 22.5 ng/g in tissue samples, respectively.

2.7 Sampling

Male Sprague-Dawley (S-D) rats were obtained from Charles River Laboratories (Raleigh, NC, USA). The rats were acclimated for at least 7 days in an AAALAC-approved

animal care facility after arrival. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia. Rats (264±4g, n=3) were dosed orally with 1 mg TCE/kg body weight using Alkamulus as the vehicle and sacrificed by cervical dislocation 10 min postdosing. Blood samples were collected immediately via cardiac puncture. The liver, kidney, lungs and brain were perfused in situ with cold saline to remove as much as blood as possible. Each tissue specimen was weighed and homogenized with two volumes of cold deionized water. Blood samples were analyzed immediately, and tissue samples were stored at −20 ºC until analysis.

3. RESULTS AND DISCUSSION

3.1 Optimization of the HS-SPME conditions

For the sampling of TCE from biological matrices, headspace (HS)-SPME was preferred to direct sampling for several reasons: (1) the headspace mode protected the fiber coating from damage by high molecular mass compounds present in the sample matrix, such as proteins; (2) the headspace allowed modification of the matrix, such as changing the pH, without damaging the fiber; (3) the equilibrium times were generally shorter with the headspace mode than with direct extraction [35, 36]. HS-SPME is an equilibrium technique. During extraction, the analyte migrates among the three phases (the aqueous matrix, the headspace above the sample and fiber coating) until equilibrium is reached. Therefore, optimization of several parameters that affect the equilibrium is of critical importance. These parameters include sample preheating time, extraction time and temperature, sample pH, salt concentrations and desorption time and temperature. All determinations were performed in duplicate, and the average values were reported. For the analysis of lower molecular weight non-polar analytes like TCE there are two choices for SPME fibers the 100-µm PDMS and 75-µm Carboxen-PDMS. The Carboxen fibers

showed enhanced response for TCE (2-3x increase) but required a much higher desorption temperature (300 ºC) when compared with PDMS fibers. In addition, the equilibration time with Carboxen fibers were nearly 3-fold longer and the repeatability was 20-30% lower than the PDMS fibers. Many of these same observations have been previously reported [37]. One additional note was that the peak shape for TCE showed tailing when using the Carboxen fiber even at high desorption temperature of 300 ºC. Therefore, the commercially available 100-µm PDMS fiber was chosen for this study, based upon its nonpolar properties, high affinity, higher precision and shorter equilibration times for TCE [29].

Extraction temperature and time are fundamental parameters for HS-SPME. According to SPME theory the fiber equilibration process is exothermic, and any increase in sampling temperature will decrease both analyte recovery and equilibrium extraction time [38, 39]. The headspace/sample partition coefficient of the analyte increases with an increase in temperature, while at the same time the fiber coating/headspace partition coefficient decreases. Each biological matrix was tested over a range of temperatures (30-60 ºC) (n=4) and extraction times (1-20 min) (n=7) to determine the optimal extraction conditions for TCE. Figure 3.1 shows the effect of extraction temperature and time on peak areas of TCE in liver homogenate. Comparison of the extraction time profiles obtained at different temperatures reveals that higher sampling temperature will increase the speed of equilibrium but decrease the amount of extracted analyte on the fiber, due to the decreased fiber coating/headspace partition coefficient. The TCE extraction process is exothermic as demonstrated by the correspondingΔH value reported in the literature. Therefore, by increasing extraction temperature there is a depletion in extraction capacity of the fiber for TCE as reported by Pawliszyn [39]. For liver homogenate, the equilibrium status can be reached within 20 min at 30 ºC. For kidney and lung tissues, 30 ºC was

also the optimal temperature for SPME extraction, but a shorter extraction time (15 min) was required to reach equilibrium. The liver contains high levels of cytochrome P450s and other heme-containing proteins, so relatively high binding of TCE would be expected. A longer extraction time was therefore needed for TCE to be transferred from the liver matrix to the headspace. For the blood samples, the addition of sulfuric acid lysed the blood cells and lowered the binding of TCE to plasma proteins. Thus 5 min at 30 ºC was found to be long enough to reach equilibration. Brain tissue behaves similarly to blood. Ionic solutes present in brain tissue help to decrease the equilibrium time and increase the extraction efficiency. Therefore, an extraction time of just 5 min was needed.

As it is known that salting can increase or decrease the amount of analyte extracted, the influence of salt on the extraction efficiency of SPME for TCE in different matrices was studied. The presence of salt increases the ionic strength of a solution and often affects the solubility of analytes in biological samples. The effect of five types of salt solutions was studied (Figure 3.2), including sodium fluoride, sodium chloride, sodium sulfate, sodium carbonate and ammonium sulfate. With liver homogenate as an example, optimization results revealed that a different concentration of each salt was required for the highest sensitivity: 10% for NaCl, 30% for $(NH_4)_2SO_4$, 2% for NaF, 15% for Na_2SO_4 and 2% for Na_2CO_3. When the salt concentration exceeded the optimum value, a decrease in sample response was observed, because analytes contributing to electrostatic interactions in the aqueous phase lose their mobility and mass transfer towards the extracting phase. When the solution is saturated by salt, the presence of a solid phase could interact with the analytes and consequently decrease the amount extracted. In the liver tissues, the use of NaCl resulted in the lowest signal. Both $(NH_4)_2SO_4$ and NaF produced high responses due to their increased ionic strength. The F^- ion enhances the ionic

strength of the solution due to the much lower dimension of F^- ion with respect to the Cl^- ion. The consequence is an increase in the salting-out effect because the water is coordinated stronger by F^- ion versus Cl^-. The effect of $(NH_4)_2SO_4$ addition can be explained by the fact that NH_4^+ is a weak base and therefore the ionic strength of the solution is increased due to hydrolysis effects. Furthermore, $(NH_4)_2SO_4$ is a divalent ion which are usually more effective than univalent ions (e.g., NaCl) for salting-out. Similarly, CO_3^{2-} is also an ion resulting from dissociation of a weak acid. However, the addition of Na_2CO_3 does not provide higher responses versus $(NH_4)_2SO_4$ due to its production of CO_2 bubbles in the matrices which lowers the mass transfer of TCE towards the fiber. In liver, lung and kidney homogenates, the highest sensitivity was obtained following addition of 30% ammonium sulfate solution. For brain, lower concentrations of salt (e.g. 5% of sodium chloride solution) worked better, because more ionic solutes are already present in brain tissue. Therefore, 30% $(NH_4)_2SO_4$ solution was selected for liver, kidney and lung, and 5% NaCl solution was chosen for brain. For blood samples, salt solutions did increase the sensitivity to some extent, but the sample response was not linear. It is likely this phenomenon resulted from partitioning of TCE into erythrocytes [40]. Salt solutions are not strong enough to lyse these cells. Sulfuric acid (1 mol/L) was therefore selected to release TCE from erythrocytes and thus increase the extraction efficiency.

Preheating time was another parameter that affected the equilibrium conditions. At low extraction temperatures, samples need to be preheated for some time before the fiber was exposed to the headspace for extraction. This preheating process improved the mass transfer kinetics of the analyte from the liquid sample to the headspace and shortened the equilibration time. Following addition of the optimized concentration of salts, each matrix was preincubated at 30 ºC in the agitator for 0, 1, 3, 5, 8, 10, 15, 20 min and extracted using the optimized extraction

conditions. From 0-5 min, an increase in preincubation time resulted in increased TCE response. Longer preheating times did not appear to improve the recovery. Thus, the shortest preheating time of 5 min was selected for this study.

Finally, the desorption time and temperature were optimized. Ideally the time interval required for desorption should be as short as possible and carryover effects should be avoided. This effect is normally obtained by applying the highest possible temperature that does not damage the fiber. The desorption temperature was examined over a range from 100-270 ºC and the desorption time recorded from 15-120 seconds. When the injection port was maintained at 150 ºC or less, a split or tailing of the chromatographic peak was observed. When higher temperatures (e.g. 250 or 270 ºC) were applied, the sensitivity no longer increased, but coatings on the fiber were removed, resulting in shorter fiber life times. Therefore, 200 ºC was selected as the optimum desorption temperature. At this temperature, 120 seconds were found to be sufficient for optimum recovery and complete analyte desorption. No carry-over was found, even following analysis of blood and tissue samples spiked with large amounts of TCE (1µg/ml). Table 3.1 summarizes the HS-SPME conditions for each matrix.

3.2 Validation of the method

After optimization the methodology was validated according to internationally accepted criteria [34]. The parameters validated were selectivity, calibration curves, precision and accuracy, limits of quantitation, recovery and stability.

The selectivity of the method was evaluated by analysis of blank matrices and matrices spiked with TCE standards. Figure 3.3 shows representative chromatograms obtained from each blank matrix and matrix spiked with the LLOQ standard (0.25 ng/ml in blood or 0.75 ng/g in tissues). No interfering peaks from endogenous compounds were observed at the retention time

of TCE. Utilization of selected-ion monitoring (SIM) mode enhanced the mass spectrometric selectivity by eliminating the need to scan a large range of masses.

Results of statistical analyses of the calibration curves for linearity are shown in Table 3.2 for different matrices. The curves were linear (R^2>0.994) over the range of 0.25 to 100 ng/ml in blood samples or 0.75 to 300 ng/g in tissue samples. SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for each matrix. The lower limit of quantitation, LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 0.25 ng/ml for TCE in blood and 0.75 ng/g for liver, kidney, lung and brain, as shown in Table 3.3. A signal-to-noise (S/N) ratio > 10 at the LLOQ was observed for TCE in all matrices. These limits were substantially lower than published values for TCE determination by SPME or by liquid-liquid extraction from blood, liver, kidney and lung [19, 30]. These LLOQs were similar to the values obtained when purge and trap method were utilized, or TCE levels were measured in other matrices such as urine. However, our results were obtained using a considerably smaller sample volume [16, 28] (e.g. 200 μL versus 2-10 ml). A procedure for determination of TCE in the brain has not previously been published.

Assay precision and accuracy for TCE in each matrix were established at the LLOQ, low, medium and high concentrations over 3 days. Table 3.3 summarizes the accuracy and precision data that were collected. The intra-day precision and accuracy (n=5) were less than 6.54% and 9.36% for TCE in all matrices. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were less than 9.21% and 9.52%.

While recovery studies are normally performed in bioanalytical method validation the values are not normally reported for SPME studies. However, relative recovery is a good parameter for evaluating the matrix effect during method validation. The relative recoveries for TCE were calculated by comparing the amount extracted by HS-SPME from different biological matrices with the amount extracted from water. The values obtained are summarized in Table 3.4. Relative recoveries for TCE from all matrices ranged from 54.5-65.5%. Biological matrices are complex. They are rich in proteins and other bio-macromolecules. The mass transfer of TCE from the aqueous phase to the headspace may be hindered from such matrices. Interestingly, relative recoveries did not vary significantly among matrices. For TCE, the viscosity of the matrices was a key point for extraction recovery and sensitivity. When the salt solutions were added to the tissue homogenates, the viscosities of different matrices were almost the same. TCE is not a highly protein bound compound, so liver and blood, which contain high concentrations of proteins, do not demonstrate lower recoveries than other tissues.

Stability testing is very important for validated methods for analysis of biological samples. A sample may remain in the autosampler for hours. TCE is a volatile compound, so examining its loss during the sample analysis is critical. Autosampler stability was evaluated at 7.5 ng TCE/ml in blood and 22.5 ng TCE/g in tissue samples. Blank blood and tissue homogenates were spiked with TCE and left in the autosampler at room temperature for 8-24 hours. These samples were compared with samples prepared freshly. The values obtained for precision were less than 6.64%, and the relative error was less than 14.22% (see Table 3.5). All matrices were stable in the autosampler for 24 hours, except blood which was only stable for 8 hours due to coagulation. Freeze and thaw stability was also evaluated for tissue homogenates at the same concentration (n=5). Blood samples were analyzed immediately, so freeze and thaw

stability testing was not necessary. Tissue homogenates were spiked with 0.75 ng/g of TCE, and these aliquots were stored at -20 ºC for 24 hr. After three complete freeze and thaw cycles, the samples were compared to those prepared freshly. The values obtained for precision and relative error were less than 8.22% and 12.92%, respectively.

3.3 Applications

To demonstrate the applicability of this HS-SPME method to toxicokinetic studies, blood and tissue samples from TCE-dosed rats were analyzed and the TCE concentration data presented in Table 3.6. Representative chromatograms from analysis of TCE in blood, liver, kidney, lung and brain 10 min after rats dosed orally with 1 mg/kg of TCE are shown in Figure 3.4. The highest TCE concentrations were found in liver, due to first-pass uptake of the chemical by the liver. TCE concentrations in kidney, lung, and brain were lower than in blood, as not enough time has elapsed for much of the lipophilic chemical to be taken up by tissues. This was similar to previously reported data for TCE in different tissues [31].

4. CONCLUSIONS

A simple, specific, rapid and very sensitive SPME-GC/MS method for the determination of TCE in various biological matrices (blood, liver, lung, kidney and brain) has been developed and validated. The technique overcomes limitations and obstacles of conventional methods such as the use of expensive and frequently contaminated organic solvents and of tedious and time-consuming sample preparation. During the SPME process, several influential parameters such as extraction time, extraction temperature, salt effect and desorption conditions were investigated and optimized for each matrix. It was clear that systematic optimization was necessary for each different biological matrix in order to enhance the extraction efficiency. This validated method yields excellent linearity, precision and accuracy over a wide calibration range and only requires

small sample volumes. The limits of quantition for this SPME-GC/MS method are 0.25 ng/ml in blood and 0.75 ng/g in tissues. To our knowledge, this is the first validated and the most sensitive SPME-GC/MS method for determination of TCE in blood and tissues of laboratory animals. This method was successfully used to quantify the blood and tissue distribution of TCE following administration of the lowest oral dose of the VOC for which the toxicokinetics have been characterized.

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Table 3.1. Summary of HS-SPME conditions for determination of TCE from rat blood, liver, kidney, lung and brain.

	Preheat time (min)	Incubation Temp (ºC)	Extraction time (min)	Desorption Temp (ºC)	Desorption Time (min)	Salt effects
Blood	5	30	5	200	2	1 M H_2SO_4 solution 400 µl
Liver	5	30	20	200	2	30% $(NH_4)_2SO_4$ solution 200 µl
Kidney	5	30	15	200	2	30% $(NH_4)_2SO_4$ solution 200 µl
Lung	5	30	15	200	2	30% $(NH_4)_2SO_4$ solution 200 µl
Brain	5	30	5	200	2	5% NaCl solution 200 µl

Table 3.2. Statistical data for linearity assessment including standard deviation (S.D.) for TCE from each matrix over three days. (Concentrations of calibration curves in blood: 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 ng/ml; concentrations of calibration curves in tissues: 0.75, 1.5, 3.0, 7.5, 15.0, 30.0, 75.0, 150.0, 300.0 ng/g.)

	Blood	Liver homogenate	Kidney homogenate	Lung homogenate	Brain homogenate
R^2	0.9947 ± 0.0034	0.9955 ± 0.0036	0.9982 ± 0.0008	0.9975 ± 0.0024	0.9954 ± 0.0019
Slope	2890 ± 330	1010 ± 220	1130 ± 160	1700 ± 60	1770 ± 200

Table 3.3. The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the HS-SPME-GC/MS method used to quantitate TCE in rat blood, liver, kidney, lung and brain.

	Intra-day			Inter-day		
Concentration TCE added	Concentration TCE found ±S.D.	R.S.D. (%)	Error (%)	Concentration TCE found ±S.D.	R.S.D. (%)	Error (%)
Blood (ng/ml)						
0.25	0.26 ± 0.01	1.62	2.48	0.25 ± 0.01	3.57	2.87
0.75	0.72 ± 0.03	3.54	4.22	0.73 ± 0.03	3.92	3.29
7.5	7.22 ± 0.34	4.70	4.02	7.52 ± 0.36	4.79	3.44
75	70.56 ± 2.32	3.29	5.92	76.23 ± 5.48	7.18	5.78
Liver homogenate (ng/g)						
0.75	0.73 ± 0.04	5.67	5.71	0.74 ± 0.07	9.21	7.22
2.25	2.04 ± 0.06	3.08	9.36	2.04 ± 0.10	4.87	9.52
22.5	20.83 ± 0.64	3.07	7.42	21.14 ± 1.24	5.88	7.19
225	211.20± 6.63	3.14	6.12	227.72 ± 11.06	5.06	4.30
Kidney homogenate (ng/g)						
0.75	0.75 ± 0.05	6.54	4.86	0.77 ± 0.05	6.19	5.45
2.25	2.20 ± 0.07	3.01	3.09	2.20 ± 0.07	2.96	2.95
22.5	23.15 ± 0.90	3.90	2.98	22.51 ± 0.90	4.00	2.91
225	238.10 ± 10.71	4.50	5.82	230.21 ± 9.46	3.15	3.30
Lung homogenate (ng/g)						
0.75	0.74 ± 0.05	6.36	4.48	0.77 ± 0.05	5.96	4.76
2.25	2.24 ± 0.16	6.08	4.30	2.23 ± 0.13	5.88	4.88
22.5	21.23 ± 0.78	3.66	5.75	21.95 ± 1.70	7.73	6.86
225	207.71 ± 1.71	0.82	7.69	222.43 ± 15.28	6.87	6.20
Brain homogenate (ng/g)						
0.75	0.79 ± 0.02	1.98	5.69	0.78 ± 0.05	7.07	6.47
2.25	2.23 ± 0.13	5.68	4.95	2.19 ± 0.12	5.67	4.47
22.5	21.33 ± 0.89	4.15	5.18	23.20 ± 1.81	7.78	7.52
225	205.32 ± 7.39	3.60	8.74	225.41 ± 20.51	9.10	7.74

Table 3.4. The relative recovery (%) (mean ± S.D.) respect to deionized water of TCE from rat blood, liver, kidney, lung and brain homogenates (n=5)

Concentration (ng/ml or ng/g)	Blood	Liver homogenate	Kidney homogenate	Lung homogenate	Brain homogenate
0.25 or 0.75	54.5 ± 1.23	63.4 ± 2.71	65.5 ± 1.47	62.3 ± 3.26	65.5 ± 1.00
0.75 or 2.25	61.0 ± 2.68	59.8 ± 5.81	61.9 ± 2.17	57.3 ± 1.30	61.7 ± 0.63
7.50 or 22.5	56.5 ± 1.51	58.5 ± 4.69	58.4 ± 3.77	55.1 ± 0.78	64.5 ± 1.17
75.0 or 225	62.3 ± 3.96	55.4 ± 3.77	55.0 ± 1.31	58.2 ± 0.62	57.4 ± 2.27

Table 3.5. Stability testing of TCE in rat blood, liver, kidney, lung and brain homogenates, (n=5)

Stability	Spiked conc. (ng/ml or ng/g)	Observed conc. ± S.D. (ng/ml or ng/g)	R.S.D. (%)	Relative error (%)
Blood				
Autosampler stability (8h)	7.5	7.03 ± 0.40	5.71	-6.32
Liver homogenate				
Three freeze-thaw cycle	22.5	23.46 ± 1.11	4.78	4.26
Autosampler stability (24h)	22.5	20.07 ± 0.60	2.96	-10.79
Kidney homogenate				
Three freeze-thaw cycle	22.5	23.85 ± 1.47	6.11	6.03
Autosampler stability (24h)	22.5	20.31 ± 0.93	4.59	-9.78
Lung homogenate				
Three freeze-thaw cycle	22.5	25.41 ± 2.10	8.22	12.92
Autosampler stability (24h)	22.5	19.29 ± 1.98	6.64	-14.22
Brain homogenate				
Three freeze-thaw cycle	22.5	20.70 ± 1.26	6.05	-8.05
Autosampler stability (24h)	22.5	19.74 ± 0.99	5.04	-12.23

Table 3.6. TCE concentrations 10 min postdosing in tissues of S-D rats dosed orally with 1 mg TCE/kg body weight

	Concentration in Rat A	Concentration in Rat B	Concentration in Rat C	Average Concentrations (ng/ml ± SD)
Blood	1.60	2.79	6.74	3.71 ± 2.69
Liver	11.67	16.73	6.54	11.65 ± 5.09
Lung	0.40	2.62	0.93	1.32 ± 1.16
Kidney	0.35	3.94	0.26	1.52 ± 2.10
Brain	0.99	2.08	2.67	1.91 ± 0.85

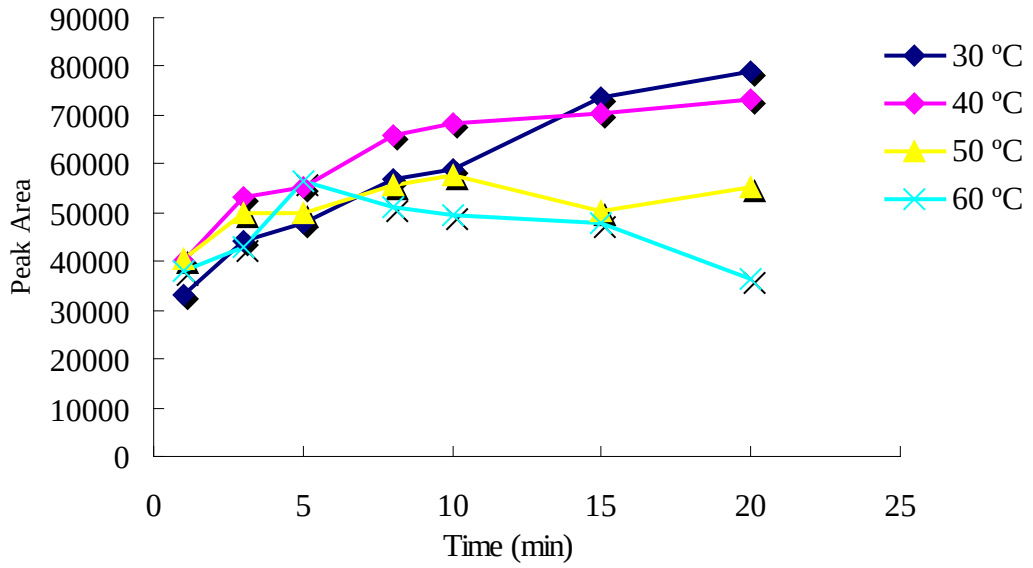

Figure 3.1. Plot of TCE peak area versus extraction time obtained on different extraction temperature (30, 40, 50 and 60 ºC) in liver tissues. Conditions: preheat 5 min, 200 µl of $(NH_4)_2SO_4$ solution added.

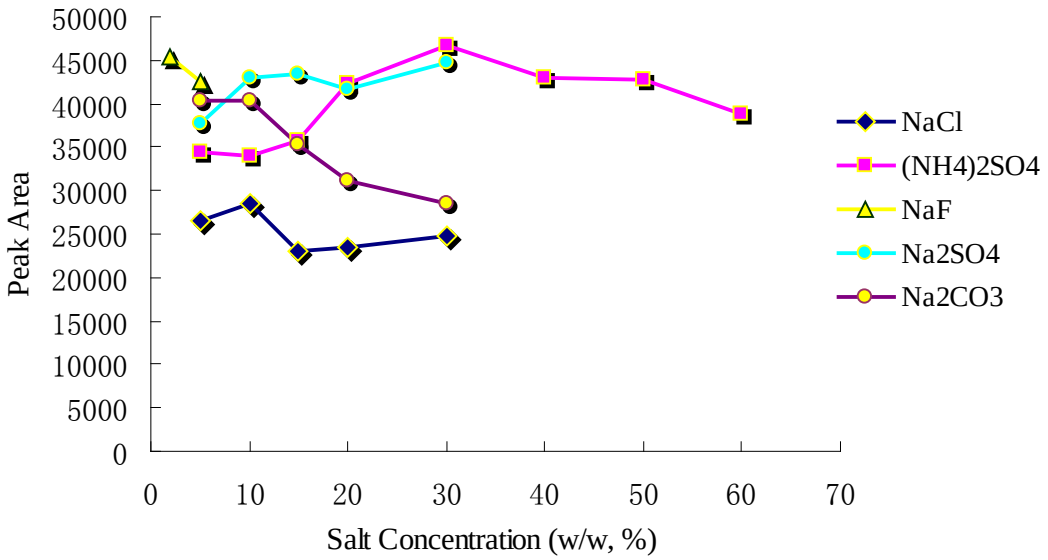

Figure 3.2. Plot of TCE peak area versus salt concentrations added into liver homogenate.

Conditions: preheat 5 min, extraction time 20 min, 30 ºC.

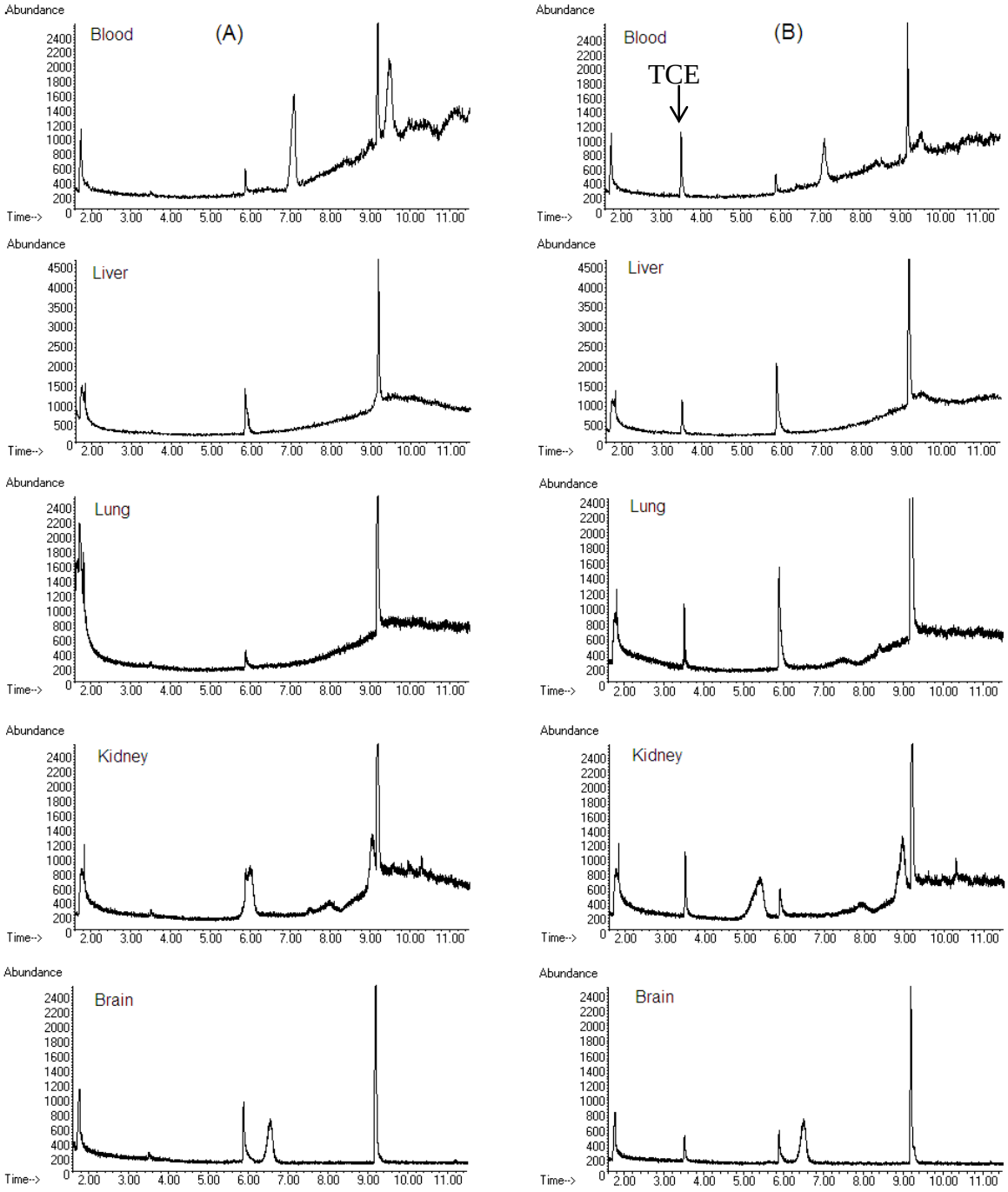

Figure 3.3. Representative chromatograms using selected-ion monitoring (SIM) mode by monitoring m/z 130 obtained from (A) blank blood, liver, lung, kidney, brain; (B) blood, liver, lung, kidney, brain spiked with the LLOQ (0.25 ng/ml in blood or 0.75 ng/g in tissues) concentration of TCE.

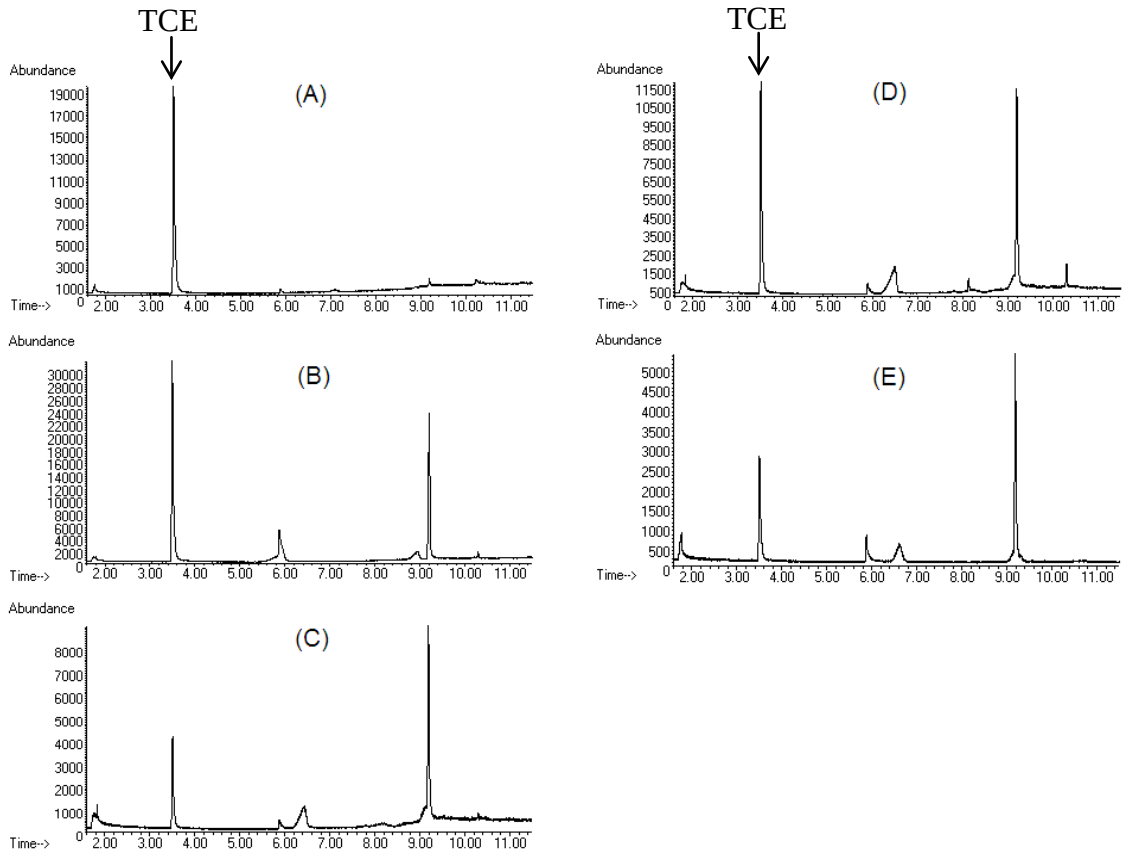

Figure 3.4. Representative chromatograms of (A) blood; (B) liver; (C) lung; (D) kidney; (E) brain samples from rats dosed with 1 mg/kg TCE orally (Blood and tissues were taken at 10 min post dosing.)

CHAPTER 4

TRACE LEVEL DETERMINATION OF TRICHLOROETHYLENE IN BIOLOGICAL SAMPLES BY HEADSPACE SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY-NEGATIVE CHEMICAL IONIZATION MASS SPECTOMETRY

Liu Y, Muralidhara S, Bruckner JV, Bartlett MG. *Rapid Communications in Mass Spectrometry.* 2008; **22**(6):797-806. Reprinted here with the permission of publisher

ABSTRACT

A gas chromatography-negative chemical ionization mass spectrometry (GC-NCIMS) method using headspace solid-phase microextraction (HS-SPME) was developed for the determination of trichloroethylene (TCE) in blood, liver, kidney, lung and brain. The method was optimized with respect to several parameters including extraction time, extraction temperature, desorption time and salt addition. The method showed good linearity over the range of 0.025-25 ng/mL in blood and 0.075-75 ng/g in tissues with correlation coefficient (R^2) values higher than 0.99. The precision and accuracy for intra-day and inter-day measurements were less than 10%. The relative recoveries of all matrices were greater than 52%. Samples showed no significant loss during 8 hours in the autosampler and following 3 freeze-thaw cycles. Validation results demonstrated that selected ion monitoring of the ^{35}Cl and ^{37}Cl isotopes using NCI resulted in reliable and sensitive quantitation. This validated method was successfully applied to study the toxicokinetics of TCE following oral administration of extremely low doses of this potential human carcinogen to small test animals (rats).

INTRODUCTION

Solid phase microextraction (SPME) is a simple and efficient, solvent-free sample preparation technique, suitable for both qualitative and quantitative analysis of volatile and semivolatile compounds in aqueous and solid matrices[1]. Since its introduction, SPME has been widely used for analyses in different fields, such as environmental monitoring, foods, flavors, fragrances, wines, biological fluids and breath analyses[2-11]. Headspace SPME has been frequently used in biological monitoring, e.g., determination of hydrocarbons[12], polycylic aromatic hydrocarbons[13], organochlorine pesticides, polychlorinated biphenyls[14], and organophosphorus pesticides[15] from plasma, serum, or urine. In the present study, headspace (HS)-SPME was applied to the determination of trichloroethylene (TCE) in biological samples from rats including blood, liver, kidney, lungs and brain.

TCE has been used extensively as a metal degreaser, chemical intermediate, dry cleaning agent and solvent. As a result of its widespread use, TCE has been frequently identified as a contaminant of ambient air, groundwater, soil and food[16-17]. TCE in drinking water is present in trace amounts, ranging from 0.02 ug/L to 230 ug/L[18]. Extremely high, chronic doses of TCE were required to produce tumors in mice and rats. Nevertheless, exposure of humans to very low levels of TCE is of concern, primarily because of the potential carcinogenic risks[19-23]. Toxicokinetic studies have shown that TCE is rapidly absorbed and efficiently metabolized. Only ~10% of low to moderate doses of TCE is eliminated via the lungs, while more than 50% of the absorbed dose is biotransformed to its major metabolites, trichloroethanol and trichloroacetic acid[24-25]. It has been theorized that low oral doses of volatile organic chemicals (VOCs) are completely removed by presystemic, or first-pass elimination[26-27]. One study of TCE showed that significant proportions of low oral doses of TCE were removed by the liver and

lungs. The presystemic elimination of TCE was inversely related to the dose[28]. Therefore, the concentrations of TCE reaching arterial blood and body tissues are expected to be extremely low or zero for environmentally-relevant exposures. It has not been possible to directly measure the efficiency of presystemic elimination of trace doses of TCE due to the lack of an analytical method with adequate sensitivity.

One approach to enhance the detectability of TCE is to improve the sensitivity of the detector. Negative chemical ionization (NCI) mass spectrometry has increased selectivity and sensitivity for electrophilic atoms (halogens), while adding molecule-specific information[29-30]. The sensitivity of procedures using NCI is often 3 to 100-fold greater than electron capture detection (ECD) methods[31], and 2-100 times more sensitive than electron ionization (EI) mass spectrometry[32-35].

The aim of this project was to optimize SPME sampling conditions and to develop a highly sensitive HS-SPME GC-NCIMS method for quantitative analysis of TCE in blood and TCE's primary target organs (i.e. liver, kidney, lung, and brain). These biological samples can be monitored after dosing to assess the extent of first-pass elimination of TCE, and to characterize the toxicokinetics of TCE following environmentally-relevant exposures. An ideal method would also prove to be sensitive and require small sample sizes, allowing it to be used with serial blood samples taken from small animals.

EXPERIMENTAL

Chemicals and reagents

Analytical grade trichloroethylene (TCE) and ammonium sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride and sulfuric acid were obtained from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific

(Pittsburgh, PA, USA). Deionized water used in experiments was generated from a Continental deionized water system (Natick, MA, USA). Ultra high purity (UHP) helium and methane were purchased from National Welders (Charlotte, NC, USA). Alkamlus, the emulsifying agent used in preparing aqueous suspensions for the animal study, was obtained from Rhone-Poulenc (Cranbury, NJ, USA).

Gas chromatography-mass spectrometry

The analyses were carried out on an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 mass selective detector (Palo Alto, CA, USA). The GC was equipped with a 0.75 mm i.d. SPME liner. Separation of the analytes was obtained on a ZB-5MS column (Phenomenex, Torrance, CA, USA) of 30 m × 0.25 mm i.d., 0.25 μm film thickness using helium as the carrier gas (flow rate, 1 mL/min). The GC injection port and interface transfer line were maintained at 200 and 280 ºC, respectively. During the fiber desorption process, the injector was operated in the splitless mode. After 2.5 min, the split vent valve was opened to sweep any residual vapors from the liner. The oven temperature was initially held at 35 ºC for 3 min, then increased to 100 ºC at 10 ºC/min, and held for 2 min. The mass spectrometer was operated in the negative chemical ionization (NCI) mode with an electron energy of 235 eV. Ultra high purity (UHP) methane was used as the reagent gas at a flow rate of 2 mL/min. The ion source temperature was set at 150 ºC. Quantitation of TCE was performed using selected-ion monitoring (SIM) of m/z 35 and 37. A solvent delay of 1.5 min was set to protect the filament from oxidation.

Preparation of working standard and quality control solutions

A stock solution of TCE was prepared in acetonitrile to yield a final concentration of 1 mg/mL. Standard solutions for the calibration curve were prepared from the stock solution in the

following concentrations: 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 250 ng/mL. Standards used to assess precision and accuracy were prepared in acetonitrile from the stock solution in concentrations of 0.25, 0.75, 7.5 and 75 ng/mL. All stock and standard solutions were prepared fresh daily and refrigerated at 4 ºC until use.

Sample preparation

Prior to extraction, liver, kidney, lung and brain tissues were homogenized with two volumes of deionized water (w/v) using a Polytron homogenizer (Brinkmann, Westbury, NY, USA). Samples for the calibration curves and quality controls (QCs) were prepared by adding 20.0 μL of the TCE standard into 200 μL of blank blood or blank tissue homogenate. This yields calibration standard concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 25.0 ng/mL in blood and 0.075, 0.15, 0.3, 0.75, 1.5, 3.0, 7.5, 15.0, 30.0 and 75.0 ng/g in tissues. The final concentrations of QCs were 0.025, 0.075, 0.75, 7.5 ng/mL in blood and 0.075, 0.225, 2.25, 22.5 ng/g in tissues. For blood samples, 200 μL of blood and 400 μL of sulfuric acid (1 mol/L) were added into a 2.0-mL SPME vial. For liver, kidney and lung, 200-μL tissue homogenates were transferred to the 2.0 mL vials containing 200 μL of an ammonium sulfate solution (30%, w/v). Brain was treated in the same manner as liver, kidney and lung, but 200 μL of 5% NaCl solution (w/v) was used instead of 30% ammonium sulfate. All the autosampler vials were quickly sealed with PTFE-coated silicone septa and crimped aluminum caps. The vials were vortexed for 2 min and placed into the autosampler for analysis. Two blanks were run before each batch of samples to insure that there was not a detectable background for TCE before beginning.

Headspace SPME procedure

Headspace SPME sampling was performed using a 100-μm polydimethylsiloxane (PDMS) fiber (Sulpelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland). Fibers were conditioned at 250 ºC for 30 min prior to use. Sample vials were preheated in an agitator for 5 min before analysis. The SPME fiber was then exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 5 min (blood and brain), 15 min (lung and kidney) or 20 min (liver) at 30 ºC under agitation, the fiber was withdrawn into the needle and immediately desorbed at 200 ºC for 2 min into the injection port of the gas chromatograph.

Method validation

The methods were validated for linearity, recovery, accuracy and precision. Calibration curves were generated by linear regression analyses of the peak area of TCE against the concentration applying a weighting scheme of $1/x^2$. Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four QC samples. Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Relative recoveries for the blood method were calculated for spiked samples at 0.025, 0.075, 0.75 and 7.5 ng/mL (n=5) by dividing the peak area for TCE spiked in rat blood by the peak area for an equal concentration of TCE in deionized water. Relative recoveries for the tissue methods were calculated using spiked samples at 0.075, 0.225, 2.25 and 22.5 ng/g (n=5) in the same way. Because TCE is volatile, every precaution was taken to ensure it did not evaporate during analysis. Autosampler stability was evaluated over a period of 8 hours, to determine if there was any loss of signal due to the time a sample spent in the

autosampler. The freeze/thaw stability was investigated by comparing the stability of samples following three freeze/thaw cycles against freshly spiked samples. The stability testing was performed at 0.75 ng/mL in blood samples and 2.25 ng/g in tissue samples, respectively.

Sampling

Male Sprague-Dawley (S-D) rats were obtained from Charles River Laboratories (Raleigh, NC, USA). The rats were acclimated (2 rats/cage) for at least 7 days in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) approved animal care facility after arrival. All experiment protocols were approved by the Laboratory Animal Care and Use Committee at the University of Georgia. The rats were cannulated, so that serial blood samples could be taken to characterize the time-course of TCE. Briefly, each rat was anesthetized and a cannula surgically inserted into the right carotid artery. Water was provided *ad libitum*, but food was withheld during a 24-h recovery period before dosing. Food was provided 3 h after dosing. TCE was given orally in doses of 1, 0.1, 0.01 and 0.001 mg/kg using Alkamulus as the vehicle. Serial blood samples from each rat were taken 2, 5, 10, 15, 30, 60, 120, 240, 360 and 480 minutes post dosing. For the tissue distribution study, a rat was given 1 mg/kg TCE orally and sacrificed by cervical dislocation 10 min post dosing. Blood samples were collected immediately via cardiac puncture. The liver, kidney, lungs and brain were perfused *in situ* with cold saline to remove as much as blood as possible. Each tissue specimen was rapidly weighed and homogenized in two volumes of cold deionized water. Blood samples were analyzed immediately. Tissue samples were stored at −20 ºC until analysis.

RESULTS AND DISCUSSION

Optimization of the HS-SPME conditions

Different parameters that affect the SPME extraction efficiency of TCE were optimized, including: extraction time, extraction temperature, desorption time and salt addition. A commercially available 100-μm PDMS fiber was chosen for this study, based on its high affinity for TCE[10].

Each biological matrix was tested over a range of temperatures (30-90 ºC) with other method parameters held constant. Based on the peak areas obtained at different temperatures, 30 ºC was found to result in the highest recovery from all matrices. Therefore, 30 ºC was chosen as the extraction temperature.

The influence of salt addition was studied by adding 200 μL of NaCl, $(NH_4)_2SO_4$, NaF, Na_2SO_4 and Na_2CO_3 solutions in different concentrations to the spiked biological samples. For liver, lung and kidney homogenates, the highest sensitivity was obtained following addition of 30% $(NH_4)_2SO_4$ solution. A lower salt concentration (5% of NaCl solution) was optimal for brain. We speculate that this was due to the higher concentrations of ionic solutes already present in the brain. For blood samples, salt solutions did increase the sensitivity to some extent, but the sample response was not linear due to the distribution of TCE into erythrocytes[36]. Therefore, sulfuric acid (1 mol/L) was selected to release TCE from the red blood cells and thus increase the extraction efficiency and method sensitivity.

Different extraction times (1, 3, 5, 8, 10, 15, 20, 25 and 30 min) were evaluated at 30 ºC in the different matrices following the addition of the optimum concentrations of salt. For liver homogenate, equilibrium status was reached within 20 min. For kidney and lung, shorter extraction times (15 min) were required. The liver contained high levels of cytochrome P450s

and other heme-containing proteins, so relatively high binding of TCE was expected. Therefore, the longer extraction time was needed for TCE to be transferred from the liver matrix to the headspace. The addition of sulfuric acid lysed blood cells and lowered the binding of TCE to plasma proteins. Thus 5 min at 30 ºC was found to be long enough to reach equilibrium. The ionic solutes present in the brain decreased the equilibrium time and increased the extraction efficiency. Therefore, an extraction time of only 5 min was needed.

Desorption times (15, 30, 60, 90 and 120 s) were also investigated. A desorption time of 2 min was found to be sufficient for optimum recovery. No carry-over effect was observed.

Identification in Negative Chemical Ionization Mode

In the negative chemical ionization mode, no molecular peak for TCE was found. Instead, the primary fragment ions, ^{35}Cl and ^{37}Cl, were the base peaks in the mass spectrum. In this case, quantitation of TCE using chlorine ions in the NCI mode decreased some of the selectivity of mass spectrometry. Discrimination between different chlorine containing species was based solely on GC retention times. Therefore, separation of TCE and its metabolites was investigated to insure the specificity of the method. Both of the TCE metabolites, chloral hydrate and trichloroethanol, were well separated from TCE (data not shown). Figure 4.1-4.5 shows the representative chromatograms obtained from each blank matrix and matrix spiked with the LLOQ standard (0.025 ng/mL or 0.075 ng/g). No other interfering peaks from endogenous compounds were observed at the retention times of TCE.

Validation of the method

The performance characteristics of the HS-SPME GC/MS method were evaluated with respect to linearity, precision and accuracy, limits of quantitation, recovery and stability.

Table 4.1 shows the calibration curves for each day of validation in different matrices. The curves showed good linear response (R^2>0.993) over the concentration range from 0.025 to 25 ng/mL or 0.075 to 75 ng/g. SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for each matrix. The lower limit of quantitation, LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 0.025ng/mL for TCE in blood and 0.075 ng/g in liver, kidney, lung and brain as shown in Table 1. A signal-to-noise (S/N) ratio > 10 at the LLOQ was observed for TCE in all matrices. These limits were much lower than the published values for TCE determination by SPME, headspace or liquid-liquid extraction from blood, liver, kidney and lung[37-39]. Currently, the most sensitive SPME-GC/MS method is used for the determination of TCE in human urine (0.01 ng/mL). The LOQ was slightly higher for the presently-described method, but was obtained using a significantly smaller sample volume[40] (200 μL versus 2 mL). In addition, blood or tissue homogenates are more complex than urine, due to the abundance of lipids and proteins. A similar procedure for determination of TCE in brain has not yet been published.

Assay precision and accuracy for TCE in each matrix were established at the LLOQ, low, medium, and high concentrations over a period of 3 days. Table 4.2 summarizes these accuracy and precision data that were collected. The intra-day precision and accuracy (n=5) were better than 10.10% and 8.72% for TCE in all matrices. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were lower than 10.70% and 9.69%, respectively.

The relative recoveries for TCE were calculated by comparing the amount extracted by the HS-SPME method from different biological matrices with the amount extracted from water.

The values obtained are summarized in Table 4.3. Relative recoveries of TCE from all matrices ranged from 52.6 to 69.7%. Biological matrices are complex and rich in proteins and other biomacromolecules. The mass transfer of TCE from such aqueous matrices to the headspace may be hindered. Interestingly, relative recoveries did not vary significantly among matrices. For TCE, the viscosity of the matrices proved to be the key factor in extraction efficiency and sensitivity. When the salt solutions were added to the tissue homogenates, the viscosities of different matrices were almost the same. TCE is not a highly protein-bound compound. Thus liver and blood, which contain significant amounts of protein, do not demonstrate lower recoveries than other tissues.

Stability testing is very important for validated analytical methods for biological samples. During the process of validation, samples may remain in the autosampler for hours. TCE is a volatile compound, so assessing its loss during sample analysis is critical. Autosampler stability was evaluated at TCE concentrations of 0.75 ng/mL in blood and 2.25 ng/g in tissue samples. Blank blood and tissue homogenates were spiked with TCE and left in the autosampler at room temperature for 8 hr. These samples were then compared with fresh samples. The values obtained for precision were less than 8.89%, and the relative error was less than 13.96% (see Table 4.4). All matrices were stable in the autosampler for 8 hr. Freeze and thaw stability of the tissue homogenates was also evaluated at the same concentration level (n=5). Tissue homogenates were spiked with 2.25 ng/g of TCE and aliquots stored at -20 ºC for 24 hr. After three complete freeze and thaw cycles, the samples were compared to those prepared freshly. The values obtained for precision and relative error were less than 14.70% and 13.51%, respectively. According to criteria for method validation, analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of the appropriate controls[41].

Applications

This HS-SPME GC/MS method was applied to study the toxicokinetic behavior of TCE in rats given extremely low oral TCE doses. Four dosage levels (1, 0.1, 0.01, and 0.001 mg/kg) of TCE were given orally to S-D rats using Alkamulus as the vehicle. Serial blood samples were taken and analyzed immediately. Figure 4.1 (c) shows the chromatogram of TCE in blood taken 10 min after a rat was dosed orally with 1 mg/kg. TCE was well separated from other chlorine-containing compounds in blood. A large peak with a retention time of 7.20 min was identified as one of TCE's major metabolites, trichloroethanol (TCOH). This peak did not appear in TCE-spiked blood. TCE blood concentration versus time profiles for rats given 4 different doses were obtained (Figure 4.6). It was obvious that TCE was absorbed quickly, as the T_{max} was only 4-5 min. TCE was quickly distributed to body tissues, resulting in a rapid decrease in TCE concentrations in the blood. The 1 and 0.1 mg/kg rats exhibit similar elimination profiles. The complete elimination phase could not be obtained for the 0.01 and 0.001 mg/kg animals, due to the extremely low levels of TCE in their blood. Nevertheless, the profiles had absorption and distribution phases that were similar to those for the higher doses. The lowest oral dose, 0.001 mg/kg, is the amount of TCE a person would ingest daily if they drank 2 L water containing 35 ppb of the VOC. The toxicokinetics of doses this low have never been evaluated before. Therefore, this method can be used to provide new data for use in assessing potential cancer risks posed by environmentally-relevant levels of TCE.

Tissue samples from TCE-dosed rats were analyzed, and the concentration data are presented in Table 4.5. Representative chromatograms resulting from the analysis of TCE in liver, kidney, lung and brain taken from rats 10 min after dosing with 1 mg/kg of TCE are shown in Figures 4.2-4.5. The highest TCE concentrations at this early time were found in liver, which

was expected for a compound that is highly extracted from the portal venous blood and subsequently metabolized. TCE concentrations in kidney, lung, and brain are even lower than blood. This pattern was similar to previously reported tissue distribution data for higher doses of TCE[42].

CONCLUSIONS

A simple, specific, rapid and sensitive HS-SPME GC-NCIMS method for the determination of TCE in various biological matrices (blood, liver, lung, kidney and brain) has been developed and validated. This method yields excellent linearity, precision and accuracy over a wide calibration range and only requires a small sample volume. The limit of quantitation for this SPME-GC/MS method was 0.025 ng/mL of blood and 0.075 ng/g of tissue, which was 1000-fold lower than that of a headspace GC-ECD method[37] and 200-fold lower than a liquid-liquid extraction GC/MS method[38] developed in our laboratory. To our knowledge, this is the most sensitive SPME-GC/MS method for determination of TCE in blood and tissues and the first to be validated. NCI mode has not been reported for the determination of TCE, but the results presented in this paper demonstrate that it was suitable for quantitation of extremely low levels of the chemical in biological samples. This method was successfully applied to characterize the toxicokinetic behavior of TCE following oral administration of much lower doses than it has been possible to study before. The ability to work with very small sample volumes of blood made it possible to take serial samples from the same rat without compromising its health.

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Table 4.1. Statistical data for linearity including standard deviation (S.D.) for TCE from each matrix, (n=3)

	Blood	Liver homogenate	Kidney homogenate	Lung homogenate	Brain homogenate
R^2	0.9954 ± 0.0004	0.9946 ± 0.0033	0.9954 ± 0.0024	0.9938 ± 0.0022	0.9956 ± 0.0009
Slope	39591.5± 1401.7	23943.9 ± 1316.9	26317.9 ± 63.6	27946.47 ± 2186.9	46423.3 ± 6487.9

Table 4.2. The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the HS-SPME-GC/MS method used to quantitate TCE in rat blood, liver, kidney, lung and brain

	Intra-day			Inter-day		
Concentration TCE added	Concentration TCE found ±S.D.	R.S.D. (%)	Error (%)	Concentration TCE found ±S.D.	R.S.D. (%)	Error (%)
Blood (ng/mL)						
0.025	0.024±0.001	2.84	5.46	0.024±0.002	6.40	5.43
0.075	0.071±0.003	4.87	5.46	0.073±0.005	6.39	4.91
0.75	0.734±0.025	3.33	2.92	0.734±0.029	3.95	3.42
7.5	7.399±0.413	5.58	3.17	7.763±0.512	6.60	5.43
Liver homogenate (ng/g)						
0.075	0.075±0.003	3.67	2.52	0.072±0.007	9.47	6.44
0.225	0.228±0.008	3.38	2.38	0.235±0.011	4.63	5.18
2.25	2.154±0.096	4.45	4.60	2.179±0.090	4.11	4.09
22.5	20.86±0.448	2.15	7.29	21.15±0.656	3.10	6.00
Kidney homogenate (ng/g)						
0.075	0.067±0.005	6.84	7.69	0.076±0.008	10.5	9.23
0.225	0.232±0.012	5.16	5.41	0.226±0.014	6.27	4.89
2.25	2.054±0.104	5.07	8.72	2.138±0.098	4.58	5.25
22.5	21.24±0.423	1.99	5.58	22.24±1.130	5.08	4.65
Lung homogenate (ng/g)						
0.075	0.082±0.006	7.71	8.68	0.076±0.008	10.7	7.78
0.225	0.208±0.006	2.70	7.57	0.219±0.013	4.90	4.40
2.25	2.143±0.031	1.44	4.76	2.136±0.074	3.46	5.29
22.5	22.36±0.240	1.07	0.97	22.27±1.334	5.99	4.37
Brain homogenate (ng/g)						
0.075	0.078±0.006	7.57	6.25	0.072±0.007	8.92	9.69
0.225	0.227±0.007	3.01	2.58	0.236±0.014	5.69	9.70
2.25	2.054±0.143	6.94	8.70	2.080±0.140	9.16	8.78
22.5	21.89±2.220	10.1	7.64	21.85±1.964	6.54	6.23

Table 4.3. The relative recovery (%) (mean ± S.D.) of TCE from rat blood, liver, kidney, lung and brain homogenates (n=5)

Concentration (ng/mL)	Blood				
0.025	63.4 ± 1.70				
0.075	66.0 ± 3.25				
0.75	57.2 ± 2.74				
7.50	59.7 ± 4.16				
Concentration (ng/g)		Liver homogenate	Kidney homogenate	Lung homogenate	Brain homogenate
0.075		57.7 ± 2.89	62.5 ± 2.74	62.3 ± 3.26	68.6 ± 3.19
0.225		60.0 ± 1.63	58.5 ± 2.81	57.3 ± 1.30	69.7 ± 3.07
2.25		63.6 ± 1.89	53.2 ± 0.75	58.3 ± 2.39	65.4 ± 2.82
22.5		67.4 ± 1.96	52.6 ± 2.29	57.8 ± 3.31	62.7 ± 4.81

Table 4.4. Stability testing of TCE in rat blood, liver, kidney, lung and brain homogenates, (n=5)

Stability	Spiked conc. (ng/mL or ng/g)	Observed conc. ± S.D. (ng/mL or ng/g)	R.S.D. (%)	Relative error (%)
Blood				
Autosampler stability (8h)	0.75	0.67 ± 0.06	8.89	-10.06
Liver homogenate				
Three freeze-thaw cycle	2.25	2.08 ± 0.16	7.95	-7.50
Autosampler stability (8h)	2.25	1.96 ± 0.02	0.94	-12.89
Kidney homogenate				
Three freeze-thaw cycle	2.25	2.18 ± 0.32	14.7	-3.13
Autosampler stability (8h)	2.25	2.03 ± 0.09	4.59	-9.78
Lung homogenate				
Three freeze-thaw cycle	2.25	1.95 ± 0.08	3.93	-13.51
Autosampler stability (8h)	2.25	1.94 ± 0.08	4.19	-13.96
Brain homogenate				
Three freeze-thaw cycle	2.25	2.18 ± 0.21	9.78	-2.89
Autosampler stability (8h)	2.25	2.22 ± 0.13	5.90	-1.50

Table 4.5. Tissue TCE concentrations 10 min of S-D rats with 1 mg/kg of TCE

	Concentration in Rat A	Concentration in Rat B	Concentration in Rat C	Average Concentrations (ng/mL ± SD)
Blood	1.94	3.64	9.29	4.96 ± 3.85
Liver	10.45	19.28	7.40	12.38 ± 6.17
Lung	0.49	2.07	0.77	1.11 ± 0.84
Kidney	0.52	4.58	0.81	1.97 ± 2.26
Brain	0.81	1.46	2.00	1.42 ± 0.60

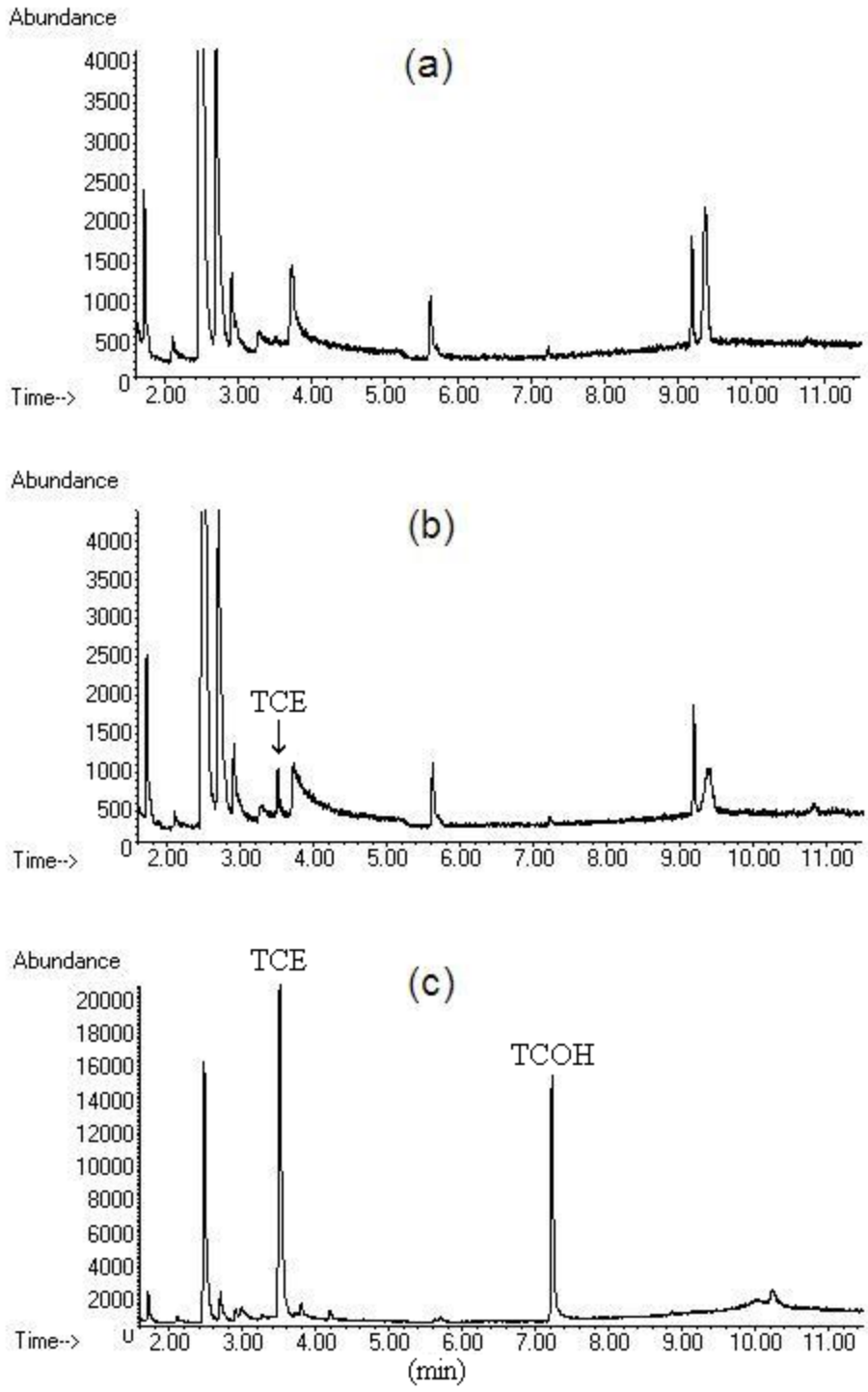

Figure 4.1. Representative chromatograms obtained from (a) blank blood; (b) blood spiked with the LLOQ (0.025 ng/mL) concentration of TCE; (c) blood sample taken 10 min after a rat was dosed orally with 1 mg TCE /kg.

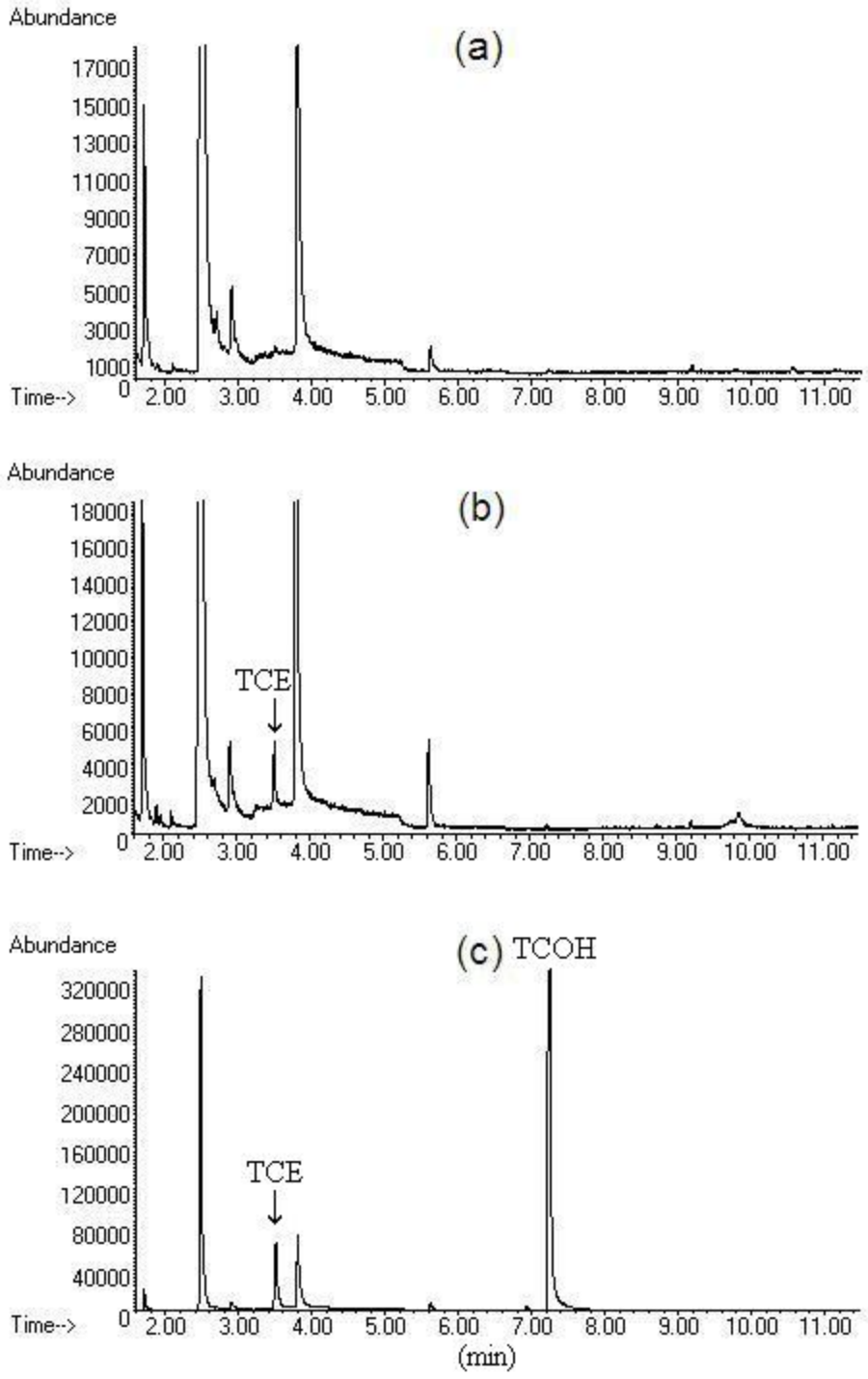

Figure 4.2. Representative chromatograms obtained from (a) blank lung homogenate; (b) lung homogenate spiked with the LLOQ (0.025 ng/mL) concentration of TCE; (c) lung tissue sample taken 10 min after a rat was dosed orally with 1 mg TCE /kg.

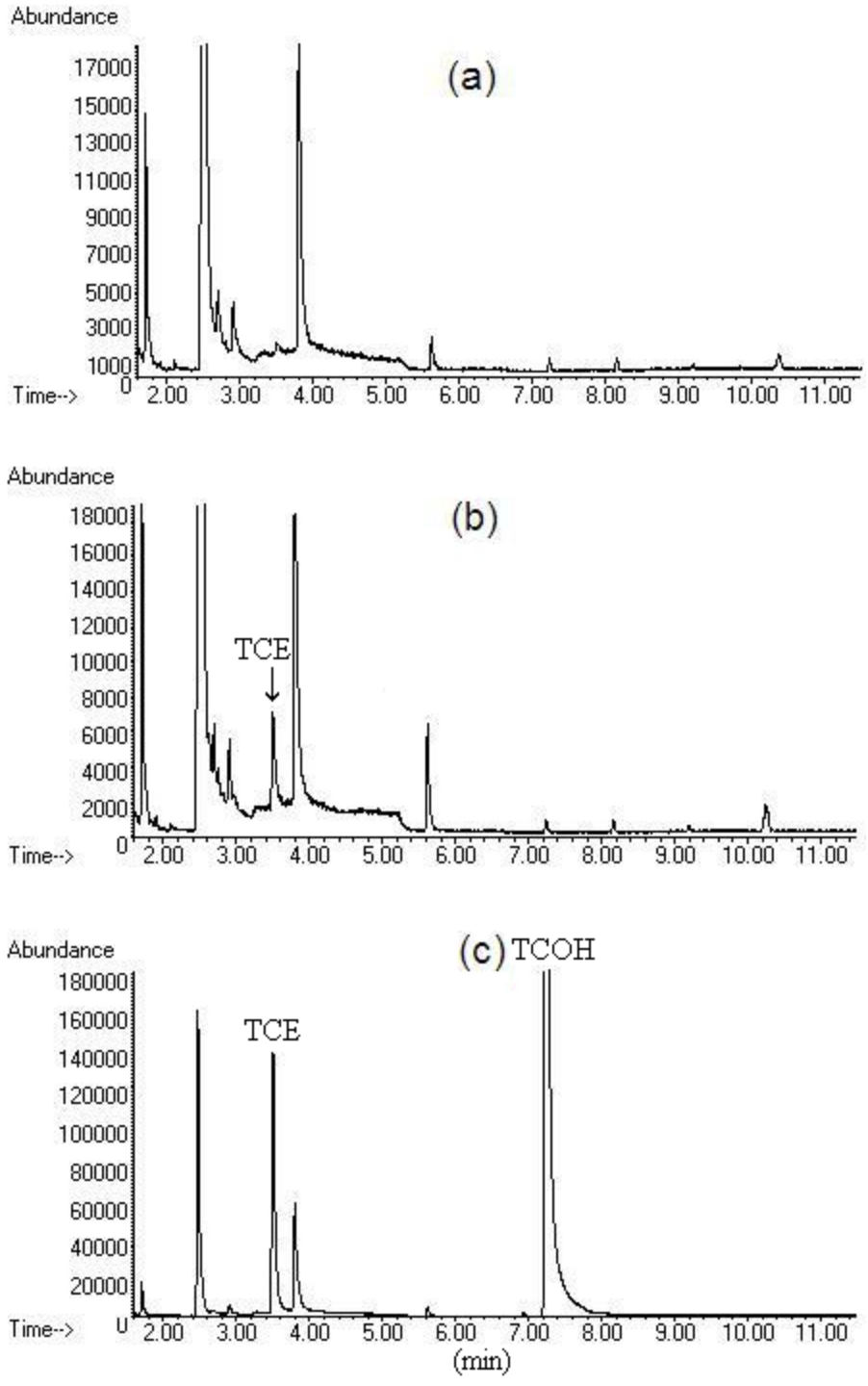

Figure 4.3. Representative chromatograms obtained from (a) blank liver homogenate; (b) liver homogenate spiked with the LLOQ (0.025 ng/mL) concentration of TCE; (c) liver tissue sample taken 10 min after a rat was dosed orally with 1 mg TCE /kg.

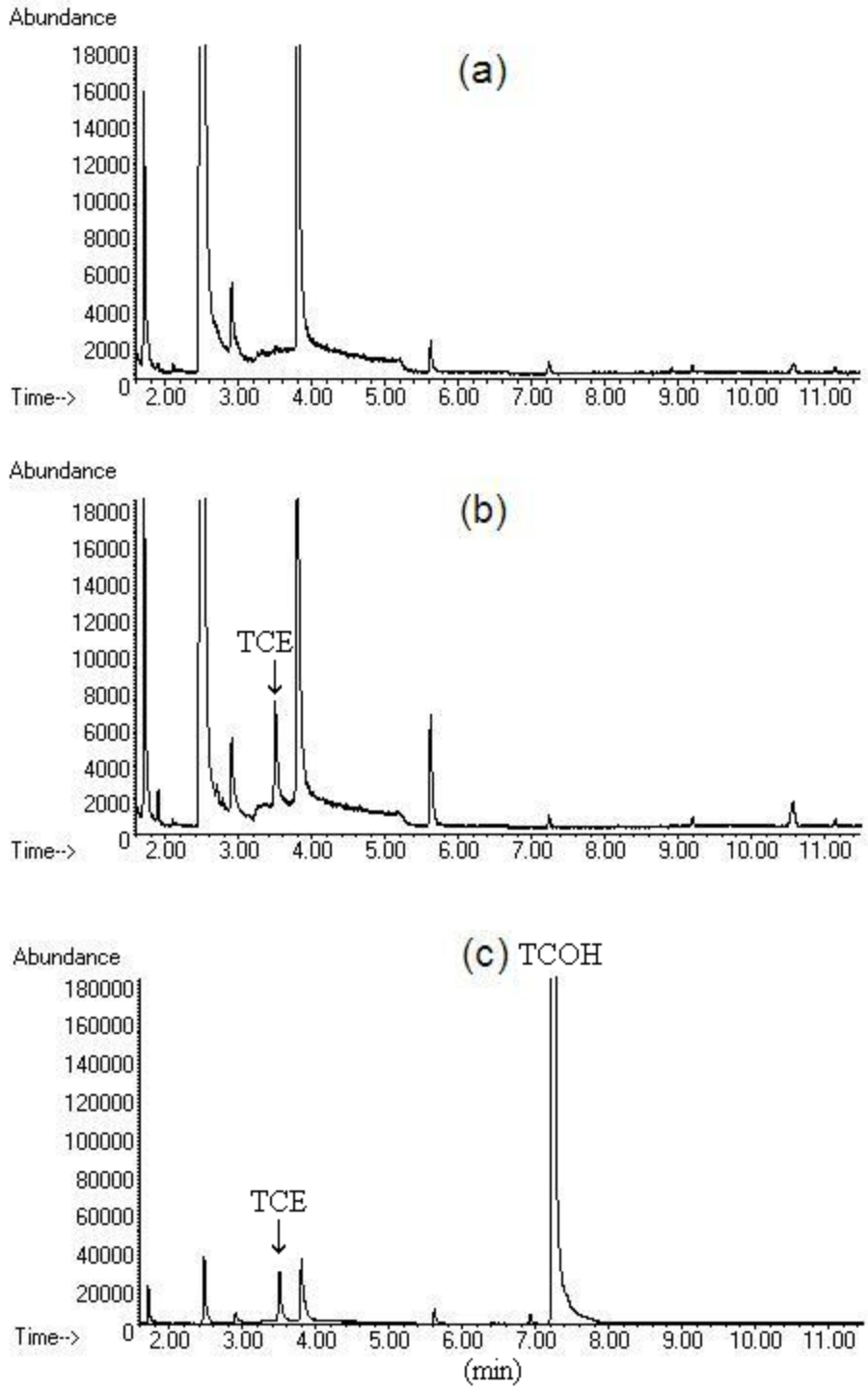

Figure 4.4. Representative chromatograms obtained from (a) blank kidney homogenate; (b) kidney homogenate spiked with the LLOQ (0.025 ng/mL) concentration of TCE; (c) kidney tissue sample taken 10 min after a rat was dosed orally with 1 mg TCE /kg.

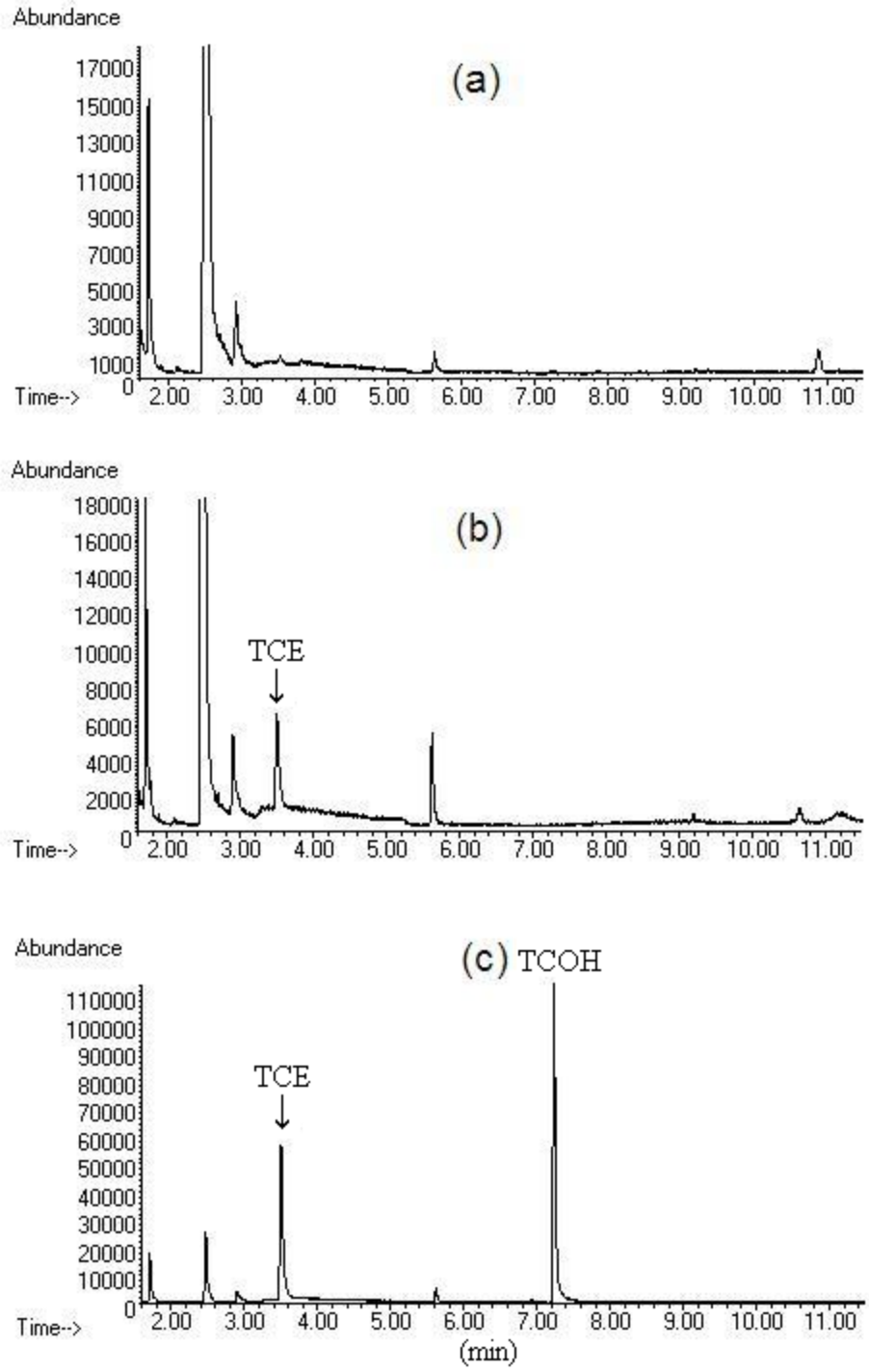

Figure 4.5. Representative chromatograms obtained from (a) blank brain homogenate; (b) brain s homogenate piked with the LLOQ (0.025 ng/mL) concentration of TCE; (c) brain tissue sample taken 10 min after a rat was dosed orally with 1 mg TCE /kg.

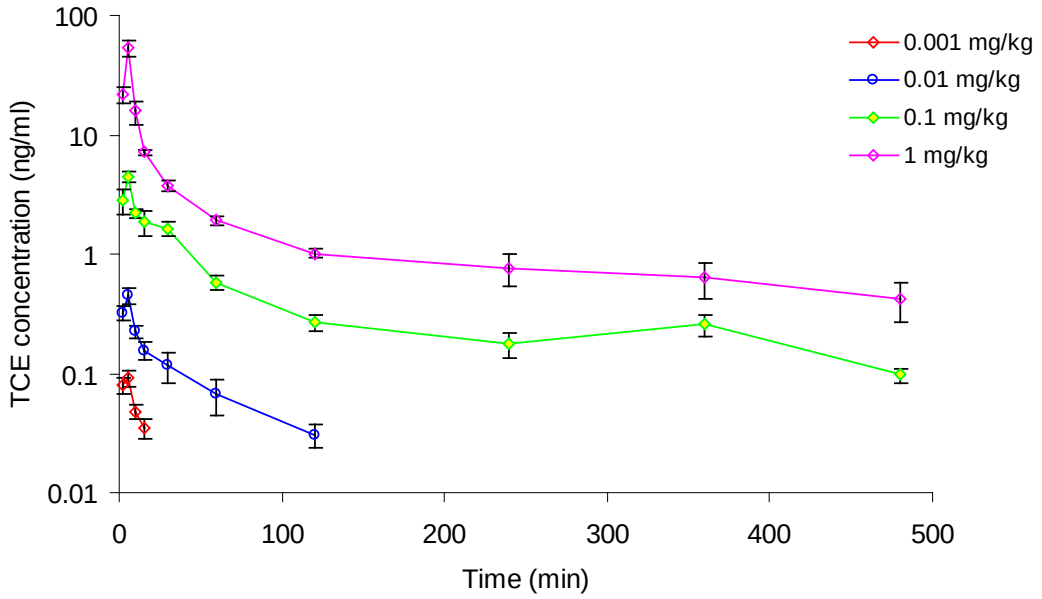

Figure 4.6. TCE blood concentration versus time profiles of S-D rats dosed orally with 0.001, 0.01, 0.1 and 1 mg/kg of TCE (mean concentration ± standard error of the mean (SEM), n=4-6 for each time point)

CHAPTER 5

DETERMINATION OF VOLATILE ORGANIC COMPOUND TRICHLOROETHYLENE FROM ADIPOSE TISSUE BY HEADSPACE SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY MASS SPECTROMETRY

Liu Y, Muralidhara S, Bruckner JV, Bartlett MG. To be submitted to *Rapid Communications in Mass Spectrometry.*

1,1,2-Trichloroethylene (TCE) is a halogenated volatile organic compound (VOC) that has been used extensively as a metal degreaser, chemical intermediate, anesthetic and dry cleaning agent. As a result of its widespread use, TCE has been frequently identified as a contaminant of ambient air, groundwater, soil and food.[1] Trichloroethylene is the most frequently detected chemical in groundwater in the proximity of more than 50% of the hazardous waste sites on the United States Environmental Protection Agency's (EPA) National Priorities List.[2-4] According to the Third National Health and Nutrition Examination (NHANES III), an estimated 10% of the US population has detectable levels of TCE in their blood.[5-6] Human come into contact with TCE most often by inhaling TCE vapor or by drinking contaminated water. TCE easily crosses the gastrointestinal wall, and is quickly distributed into the bloodstream. It is partially eliminated from the body through exhaled air and is rapidly metabolized by the liver. Unmetabolized TCE is retained in adipose tissue because of its high liposolubility.[7-8] In a study by Keys, rats were dosed orally with 8 mg TCE/kg body weight.[9] Within 30 min, there was a large accumulation of TCE in adipose tissue, with significant levels present after 4 hr and traces still visible after 20 hr. TCE and several of its metabolites, such as trichloroacetic acid and dichloroacetic acid, are known to cause a variety of health hazards. Exposure to high doses of TCE can result in central nervous system depression, liver changes and cardiac arrhythmias.[1] Exposure of the general population to very low levels of TCE in environmental media is of concern primarily because of potential carcinogenic risk.[10-13]

Methods that have been published to quantify TCE in biological samples include purge and trap, headspace, and liquid-liquid extraction.[14-18] However, none of these methods are suitable for analyzing TCE in adipose tissue due to its high liposolubility. Usually, analyzing lipophilic compounds in adipose tissue is not an easy task. Conventional methods for analyzing

these compounds in fatty samples involve several steps including extraction, elimination of the glycerophospholipids using acid digestion,[19] column adsorption chromatography,[20] solid phase extraction,[21] or normal phase liquid chromatography clean up[22-23] prior to analytical determination by GC-ECD or GC-MS. Solid phase microextraction (SPME) is an innovative, solvent-free technology that is fast, economical, and versatile. SPME can pre-concentrate the analyte on the fiber, minimize sample handling, and reduce the potential error and matrix effect. Several methods have been published describing the use of SPME for the analysis of TCE in biological sampes.[24-30] However, no method is available for determination of TCE in adipose tissue. Moreover, there is no validated analytical method published for determination of any lipophilic compounds from adipose homogenates using SPME.

The aim of this project was to optimize SPME sampling conditions and to develop a sensitive HS-SPME GC/MS method for quantitative analysis of TCE in adipose tissues. These biological samples were monitored after dosing to characterize the toxicokinetics of TCE following environmentally-relevant exposures and estimate the accumulation of TCE in adipose tissues. Both EI and NCI modes were used in this study due to their high sensitivity achieved in previous studies of the determination of TCE in other tissues.[29-30] Special treatment of the adipose samples was needed because of the high levels of lipids in the sample. The lower limit of quantitation (LLOQ) for TCE for both the EI and NCI method is 2.67 ng/g.

Analytical grade trichloroethylene (TCE) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water used in experiments was generated from a Continental deionized water system (Natick, MA, USA). Ultra high purity (UHP) helium and methane were purchased from National Welders

(Charlotte, NC, USA). Alkamlus, the emulsifying agent used in preparing aqueous suspensions for the animal study, was obtained from Rhone-Poulenc (Cranbury, NJ, USA).

The analyses were carried out on an Agilent 6890 gas chromatograph (GC) coupled with a model 5973 mass selective detector (Palo Alto, CA, USA). The GC was equipped with a 0.75 mm i.d. SPME liner. Separation of the analytes was obtained on a ZB-5MS column (Phenomenex, Torrance, CA, USA: 30m×0.25mm i.d., 0.25μm film thickness) using helium as a carrier gas (flow rate, 1 mL/min). The GC injection port and interface transfer line were maintained at 250 and 280 ºC, respectively. During the fiber desorption process, the splitless mode of injection was operated. After 2.5 min, the split vent valve opened to sweep any residual vapors from the liner. The oven temperature was initially held at 35 ºC for 3 min, then increased to 100 ºC at 10 ºC /min, and held for 2 min. The oven post run temperature was set at 280 ºC and held for 20 min. The mass spectrometer was operated in positive electron ionization (EI) mode with an electron energy of 70 eV and in negative chemical ionization (NCI) mode with an electron energy of 235 eV. In EI mode, quantitation of TCE was performed using selected-ion monitoring (SIM) of m/z 130 (quantitation ion), m/z 132 and m/z 134 (confirmation ions). In NCI mode, methane was used as the reagent gas at a flow rate of 2 mL/min. The ion source temperature was set at 150 ºC. Quantitation of TCE was performed using SIM of m/z 35 and 37. A solvent delay of 2.5 min was set to protect the filament from oxidation.

A stock solution of TCE was prepared in acetonitrile to yield a final concentration of 100 mg/ml. Standard solutions for the calibration curve were prepared from the stock solution in the following concentrations: 10, 50, 100, 500, 1000, 2500 and 5000 ng/ml. Standards used to assess precision and accuracy were prepared in acetonitrile from the stock solution in concentrations of 10, 25, 750 and 3750 ng/ml for EI mode and 10, 30, 300 and 3000 ng/ml for NCI mode. All

stock and standard solutions were refrigerated at 4 ºC during the day of use and were prepared fresh daily.

Prior to extraction, adipose samples were homogenized with two volumes of deionized water (w/v) using a homogenizer (Polytron®, Brinkman, Switzerland). Samples for the calibration curves and quality control (QC) samples were prepared by adding 20 μL of the TCE standard into 200 μL of blank adipose homogenate. This yielded calibration standard concentrations of 2.67, 13.35, 26.7, 133.5, 267, 667.5 and 1335 ng/g in adipose tissues. The final concentrations of QCs were 2.67, 6.68, 200.25, 1001.25 ng/g for EI mode and 2.67, 8.01, 80.1, 801.0 ng/g for NCI mode in adipose tissues. For the EI method, 200 μL of adipose homogenate and 400 μL of sodium hydroxide (2 mol/L) were added into a 2.0 mL SPME vial. For NCI method, 200 μL of adipose homogenate was transferred in 10.0 ml vials containing 400 μL of sodium hydroxide (2 mol/L) and 3 mL of deionized water. All the autosampler vials were quickly sealed with PTFE-coated silicone septa and crimped aluminum caps. The vials were vortexed for 3 min and placed into the autosampler for analysis. Two blanks were run before each batch of samples to insure there was not a detectable background level of TCE before beginning.

Headspace SPME sampling was performed using a 100-μm polydimethylsiloxane (PDMS) fiber (Sulpelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland). Fibers were conditioned at 250 ºC for 30 min prior to use. For both the EI and NCI methods, sample vials were preheated in the agitator for 5 min before analysis, and the SPME fiber was then exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 30 min at 30 ºC under agitation, the fiber was withdrawn into the needle and immediately desorbed at 250 ºC for 2 min into the GC

injection port. After desorption, the fiber was baked at 250 ºC for another 20 min to remove the residual fatty acid compounds adsorbed on the fiber.

The methods were validated for linearity, recovery, accuracy and precision. Calibration curves were generated by linear regression analyses of the peak area of TCE against the concentration applying a weight ($1/x^2$). Precision (expressed as % relative standard deviation, %RSD) and accuracy (expressed as %error) were calculated for four QC samples. Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Relative recoveries from adipose tissues were calculated for spiked samples at 2.67, 6.68, 200.25, 1001.25 ng/g (n=5) for the EI method and at 2.67, 8.01, 80.1, 801.0 ng/g (n=5) for the NCI method by dividing the peak area for TCE by the peak area for an equal concentration of TCE in deionized water. Because TCE is volatile, every precaution was taken to ensure it was not lost during analysis. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls.[31] The autosampler stability was evaluated over a period of 24 hours to determine if there was any loss of signal due to the time a sample spent in the autosampler prior to analysis. The freeze/thaw stability was investigated by comparing the stability of samples following three freeze/thaw cycles against freshly spiked samples. The stability testing was performed at 267 ng/g in adipose tissue samples.

Male Sprague-Dawley (S-D) rats were obtained from Charles River Laboratories (Raleigh, NC, USA). The rats were acclimated for at least 7 days in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal care facility after arrival. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Rats (264±4g, n=3) were dosed orally with 1

mg TCE/kg body weight using Alkamulus as the vehicle and sacrificed by cervical dislocation 10 min postdosing. The adipose tissues were perfused *in situ* with cold saline to remove as much blood as possible. The intact adipose tissues were stored at −20 ºC until analysis. Prior to analysis, each tissue specimen was weighed and homogenized with two volumes of cold deionized water.

Different parameters that affect the SPME extraction efficiency of TCE were optimized, including: extraction time, extraction temperature, desorption time and salt addition. A commercially available 100-μm PDMS fiber was chosen for this study, based on its high affinity for TCE reported in earlier studies.[28-30]

It is reported that salt solutions present in the biological matrices, such as plasma, liver, kidney, lung and brain homogenate, could increase the amount of TCE extracted by the fiber.[28-30] However, salting had no significant effect on the extraction efficiency of TCE in adipose tissues. Sodium hydroxide was then used to catalyze the hydrolysis of lipids in the adipose tissues. As long chain fatty acids were released, the fiber coating/sample distribution constant was increased. According to the principles of SPME,[32] the amount of TCE extracted by the fiber would increase with a higher fiber coating/sample distribution constant. Different concentrations of sodium hydroxide (1 to 6 mol/L) were studied. These results revealed that 400 μL of 2 mol/L sodium hydroxide was the optimal concentration for this study.

According to the previous SPME studies of TCE, [28-30] 30 ºC was used as the extraction temperature. Different extraction times (10, 20, 30, 40 and 50 min) were evaluated. TCE is a nonpolar compound which prefers to distribute in adipose tissue requiring long extraction times for equilibrium to be reached. In adipose homogenate, 30 min was observed to be sufficient to transfer TCE from the matrix to the headspace and then to the fiber. Longer extraction times,

(e.g., 40 min or 50 min) showed no further improvement on extraction efficiency. Using 30 min as the extraction time, different extraction temperatures (30, 40, 50 and 60 ºC) were tested. Higher extraction temperatures would increase the speed of equilibrium but decrease the amount of extracted analyte on the fiber, due to the decreased fiber coating/headspace partition coefficient. Therefore, it was not surprising that 30 ºC was found to be the optimal extraction temperature.

The desorption temperature was examined over a range from 100-270 ºC and the desorption time was recorded from 15-120 seconds. Compared with tissue homogenates from other organs, higher desorption temperatures were needed for adipose tissues because fatty acids from the matrix with high boiling points could also be extracted on the fiber.[28-30] The optimum temperature for TCE and fatty acids to be desorbed was 250 ºC. At this high temperature, 2 min was found to be long enough for TCE desorption and no carry over effect was observed. However, under these desorption conditions, less TCE was extracted by the fiber in subsequent HS-SPME procedures due to the residual fatty acids on the fiber. Therefore, an improved clean-up procedure for the fiber was essential. At the recommended conditioning temperature for PDMS fibers (250 ºC), 20 min of heating was found to be necessary for the fiber to be reused.

Similarly, the fatty acids left in the gas chromatography column also needed to be eliminated after each run. The post run temperature for the oven was set 280 ºC and the post run time was 20 min. Experimental results demonstrated that this post run processing was critical for this method to behave in a reproducible manner. Without the fiber clean-up program and the post column clean-up procedure, the performance of the column was irreversibly altered after 5-10 injections.

Both the EI and NCI methods were validated according to internationally accepted criteria.[31] The parameters validated were selectivity, calibration curves, precision, accuracy, limits of quantitation, recovery and stability.

The selectivity of both methods was evaluated by analysis of blank adipose homogenate and adipose homogenate spiked with TCE standards. Figure 5.1 shows representative chromatograms obtained from blank matrix and matrix spiked with the LLOQ standard (2.67 ng/g in adipose tissue) in EI mode. No interfering peaks from endogenous compounds were observed at the retention time of TCE. Figure 5.2 shows representative chromatograms obtained from blank adipose homogenate and adipose spiked with the LLOQ standard (2.67 ng/g in adipose tissues) in NCI mode. Because no molecular peak for TCE was found in NCI mode, the chlorine isotope ions were used for quantitation of TCE which decreased some of the selectivity of mass spectrometry. More peaks were present in the chromatograms when compared with the EI method, but no other interfering peaks from endogenous compounds were observed at the retention time of TCE.

The calibration curves generated for the validation of TCE showed a linear response (R^2>0.994) over the range from 2.67 to 1335 ng/g for both methods. SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for each matrix. The lower limit of quantitation, LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 2.67 ng/g for TCE in both methods, as shown in Table 5.1. NCI mass spectrometry usually has increased sensitivity for electrophilic atoms (halogens).[33-34] The sensitivity of procedures using NCI is often 2-100 times more sensitive than EI mass spectrometry.[35-38] In our previous study, NCI increased the sensitivity of TCE 10 fold

when using the same SPME conditions.[29] Therefore, we expected the LLOQ of the NCI method to be 10 fold lower than EI. However, direct transferring of the EI method to NCI did not yield an improved LLOQ. When comparing the chromatograms from Figures 5.1 and 5.2 it should be noted that the signal for TCE does increase a factor of 5 when moving from EI to NCI. However, the baseline noise also increases by the same factor creating the same signal-to-noise ratio. It should be noted that the two methods do not monitor the same m/z values. The NCI method only monitors the chlorine ions and these low mass ions appear to be sensitive to increased background presumably from lipids in the adipose tissue.

Assay precision and accuracy for TCE for both methods were established at the LLOQ, low, medium and high concentrations over 3 days. Table 5.1 summarizes the accuracy and precision data that were collected. The intra-day precision and accuracy (n=5) were less than 8.53% and 9.01% for TCE in all matrices. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were less than 10.59% and 8.59%.

The relative recoveries for TCE were calculated by comparing the amount extracted by the HS-SPME method from adipose homogenate with the amount extracted from water. The values obtained are summarized in Table 5.2. Relative recoveries of TCE for the EI method ranged from 9.06-11.3% and were much lower than other tissue homogenates (50-60% for liver, lung, kidney or brain homogenate). Adipose homogenates are rich in lipids and other biomacromolecules. TCE is a nonpolar compound so it prefers distribution in fatty acids versus water. The mass transfer of TCE from such aqueous matrices to the headspace appears to be hindered. Interestingly, relative recoveries from the NCI method were higher than the EI method, ranging from 28.6-36.5% as shown in Table 5.2. The addition of 3 mL of water was the reason.

The additional water in adipose homogenate decreased the viscosity of the matrix and reduced concentrations of lipids. Therefore, the extracted amount of fatty acids on the fiber was decreased and the matrix effect was lowered.

Stability testing is very important for validated methods for analysis of biological samples, especially for adipose tissues. Around 40 min including extraction and desorption was needed for each sample. A prepared adipose sample may remain in the autosampler for hours. Autosampler stability was evaluated at 267 ng/g in adipose tissues. Blank adipose homogenates were spiked with TCE and left in the autosampler at room temperature for 8-24 hours. These samples were compared with samples prepared freshly. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls.[31] Adipose samples were only stable in the autosampler for 8 hours. The values obtained for precision were less than 14.93%, and the relative error was less than 13.76% (see Table 5.3). Freeze and thaw stability was also evaluated at the same concentration. After the adipose homogenate was stored at -20 ºC for 24 hr, the samples were thawed and compared to those prepared freshly. From these experiments the relative error was found to be higher than 20%. Thus the adipose homogenates were considered to be unstable during freeze and thaw cycles. However, the intact adipose tissue can be frozen. The relative error was less than 15% (data not shown). Therefore, when necessary we stored adipose tissue in the freezer and prepared the homogenate prior to analysis.

To demonstrate the applicability of the HS-SPME EI-MS method and HS-SPME NCI-MS method to toxicokinetic studies, adipose tissue samples from TCE-dosed rats were analyzed and the TCE concentration data presented in Table 5.4. Representative chromatograms from analysis of TCE in adipose 10 min after the rats were dosed orally with 1 mg/kg of TCE are

shown in Figure 5.1(c) and 5.2(c). There are no significant differences in the concentrations of TCE obtained by the two methods. TCE is a lipophilic compound and is quickly distributed to body tissues. At 10 min after dosing, the highest TCE concentrations were found in liver, due to first-pass uptake of the chemical by the liver.[31] TCE concentrations in adipose tissue were slightly lower than liver tissue as not enough time had elapsed for much of this lipophilic chemical to be taken up by adipose tissues.

In conclusion, two selective and sensitive HS-SPME GC-MS (EI-MS and NCI-MS) methods for the determination of TCE in adipose homogenate have been developed and validated. The HS-SPME technique overcomes limitations and obstacles of conventional methods including tedious and time-consuming sample preparation for adipose samples. Both EI and NCI modes are suitable for analysis of TCE from adipose tissues. Both of these two validated methods yield excellent linearity, precision and accuracy over a wide calibration range. The limit of quantitation for TCE for both methods is 2.67 ng/g. To our knowledge, these are the first validated HS-SPME GC/MS methods for determination of TCE in adipose tissues from laboratory animals. These methods were successfully used to quantify the accumulation of TCE in adipose tissue following administration of a low oral dose.

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Table 5.1. The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the HS-SPME-GC/MS method used to quantitate TCE in adipose homogenate.

	Intra-day			Inter-day		
Concentration TCE added	Concentration TCE found ±S.D.	R.S.D. (%)	Error (%)	Concentration TCE found ±S.D.	R.S.D. (%)	Error (%)
Fat homogenate (ng/g) EI						
2.67	2.62 ± 0.16	5.61	4.18	2.75 ± 0.19	6.75	5.64
6.68	7.08 ± 0.51	7.17	8.91	6.76 ± 0.69	10.23	7.61
200.25	210.48 ± 11.05	5.25	6.20	200.33 ± 10.28	5.13	4.40
1001.25	994.92 ± 31.96	3.21	2.01	950.84 ± 68.19	7.17	7.11
Fat homogenate (ng/g) NCI						
2.67	2.83 ± 0.24	8.19	9.01	2.75 ± 0.29	10.59	8.30
8.01	8.52 ± 0.24	2.71	6.32	7.80 ± 0.61	8.03	6.87
80.1	85.41 ± 7.29	8.53	7.90	81.17 ± 7.72	9.51	7.58
801.0	835.42 ± 41.65	4.98	5.66	796.94 ± 79.22	9.94	8.59

Table 5.2. The relative recovery (%) (mean ± S.D.) respect to deionized water of TCE from rat adipose homogenates (n=5)

Concentration (ng/g)	EI	Concentration (ng/g)	NCI
2.67	10.3 ± 0.65	2.67	32.9 ± 3.58
6.68	11.3 ± 0.44	8.01	28.6 ± 1.74
200.25	9.25 ± 0.62	80.1	34.5 ± 3.87
1001.25	9.06 ± 0.57	801.0	31.1 ± 2.40

Table 5.3. Autosampler stability (8h) testing of TCE in rat adipose homogenates using EI and NCI method, (n=5)

Stability	Spiked conc. (ng/g)	Observed conc. ± S.D. (ng/g)	R.S.D. (%)	Relative error (%)
EI	267	303.75 ± 45.31	14.91	13.76
NCI	267	243.70 ± 36.40	14.93	-8.73

Table 5.4. TCE concentrations 10 min postdosing in adipose tissue of S-D rats dosed orally with 1 mg TCE/kg body weight

	Concentration in Rat A (ng/ml)	Concentration in Rat B (ng/ml)	Concentration in Rat C (ng/ml)	Average Concentrations (ng/ml ± SD)
EI	2.22	9.10	9.46	6.92 ± 4.08
NCI	1.99	10.87	10.20	7.69 ± 4.95

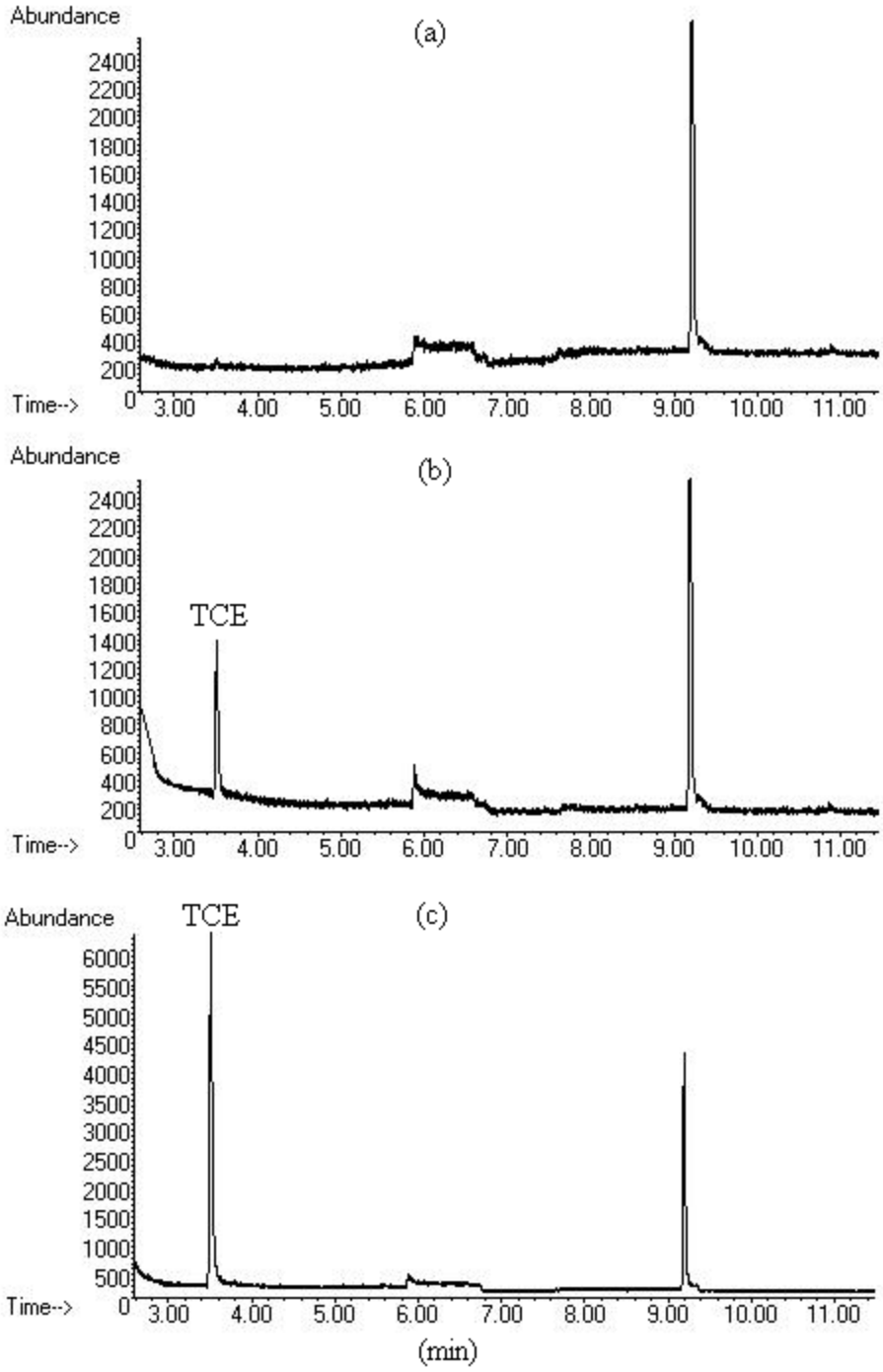

Figure 5.1. Representative chromatograms in EI mode obtained from (a) blank adipose homogenate; (b) adipose homogenate spiked with the LLOQ (2.67 ng/g) concentration of TCE; (c) adipose tissue sample taken 10 min after a rat was dosed orally with 1 mg TCE /kg.

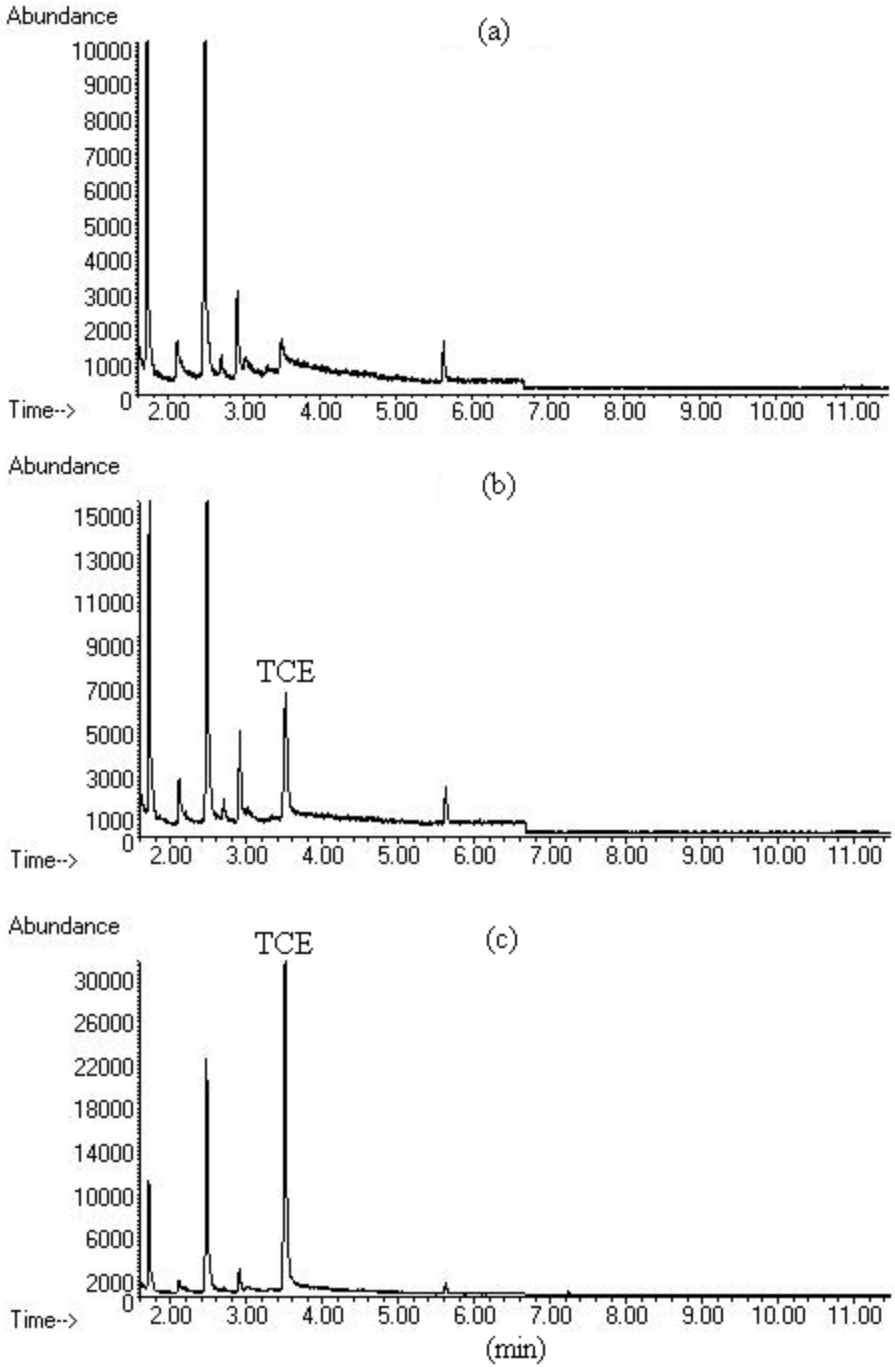

Figure 5.2. Representative chromatograms in NCI mode obtained from (a) blank adipose homogenate; (b) adipose homogenate spiked with the LLOQ (2.67 ng/g) concentration of TCE; (c) adipose tissue sample taken 10 min after a rat was dosed orally with 1 mg TCE /kg.

CHAPTER 6

IN SITU DERIVATIZATION/ SOLID-PHASE MICROEXTRACTION (SPME) COUPLED WITH GAS CHROMATOGRAPHY-NEGATIVE CHEMICAL IONIZATION MASS SPECTOMETRY FOR THE DETERMINATION OF TRICHLOROETHYLENE METABOLITES IN RAT BLOOD

Liu Y, Muralidhara S, Bruckner JV, Bartlett MG. *Rapid Communications in Mass Spectrometry.* 2008; **22**(7): 1023-1031. Reprinted here with the permission of publisher

ABSTRACT

An *in situ* derivatization solid-phase microextraction method was developed for the determination of the trichloroethylene (TCE) metabolites, trichloroacetic acid (TCA), dichloroacetic acid (DCA) and trichloroethanol (TCOH) in rat blood. The analytical procedure involves derivatization of TCA and DCA to their ethyl esters with acidic ethanol, headspace sampling using solid-phase microextraction (SPME), and gas chromatography-negative chemical ionization mass spectrometry (GC-NCI-MS) determination. Parameters affecting both derivatization efficiency and the headspace SPME procedure, such as the concentration of sulfuric acid, amount of ethanol, derivatization-extraction temperature and time, sample preheating time, agitator speed and desorption conditions, were optimized. The method showed good linearity over the range of 1-1000 ng/mL in rat blood for each metabolite with correlation coefficients (R^2) higher than 0.99. The precision and accuracy for intra-day and inter-day were less than 10%. The relative recoveries for all analytes were greater than 84%. Validation results demonstrated that selected ion monitoring of the ^{35}Cl and ^{37}Cl isotopes using NCI resulted in reliable and sensitive quantitation of all three TCE metabolites. This validated method was successfully applied to study the toxicokinetic behavior of TCE metabolites following a 1 mg/kg oral dose of TCE.

INTRODUCTION

Trichloroethylene (TCE) is a volatile organic chemical (VOC) that has been used extensively as a metal degreaser, chemical intermediate, drying cleaning agent and solvent in industry. As a result of its widespread use, TCE is frequently identified as a contaminant in ambient air, groundwater, soil and food.[1,2] According to recent nationwide surveys, approximately 10% of the US population have detectable levels of TCE in their blood.[3,4] Human exposure to TCE in environmental media is of concern, because high doses of TCE have been found to be carcinogenic in laboratory animals.[5,6] The tumors evoked by TCE are attributable to certain of its metabolites.[7] The major pathway for TCE metabolism is oxidation via the cytochrome P450 system to chloral hydrate, and subsequently to trichloroethanol (TCOH), trichloroacetic acid (TCA) and dichloroacetic acid (DCA).[5] Chloral hydrate is a drug used as a sedative prior to surgery for adults, and for children who are undergoing a clinical procedure where they must remain still, such as magnetic resonance imaging (MRI).[8,9] Recent studies of TCOH showed that TCOH can induce nephrotoxicity in rats due to its conversion to formic acid and the resulting acidosis.[10,11] It has been determined that TCA and DCA are responsible for liver tumorigenesis in mice and/or rats.[11] TCA and DCA are, in addition, frequently found in drinking water as byproducts of water chlorination. Therefore, it is important to be able to quantify these metabolites in biological specimens from exposed populations. Measurement of TCE's metabolites can play an important role in assessing the toxicokinetics and toxicodynamics of these potential human carcinogens. As TCE exposure levels are usually quite low, an analytical method with high sensitivity is required.

Different types of assays have been developed for determination of TCA, DCA and TCOH in drinking water and in biological samples[12]. The first were spectrophotometric methods

based on the Fujiwara reaction. However, these are limited by low sensitivity and specificity.[13] Numerous investigations have described both gas chromatography (GC) and liquid chromatography (LC) methods with high sensitivity and specificity for the analysis of these metabolites[14]. TCA and DCA are non-volatile, water-soluble and ionized in most solutions. LC techniques including ion chromatography have been reported for the direct analysis of TCA and DCA in plasma,[15] urine[16,17] and water.[18] These procedures have limits of detection in low $\mu g \cdot L^{-1}$ levels. However, the lack of suitable capability to simultaneously detect TCOH makes these LC methods less desirable for the study of TCE. An ion-exclusion chromatography method has been developed for direct analysis of TCOH in plasma and urine, though it has very high LOQ (limit of quantitation), 3 $mg \cdot L^{-1}$.[19] The determination of TCOH in plasma by high performance liquid chromatography-ultraviolet (HPLC-UV) method required a complicated derivatization procedures prior to analysis.[20]

GC is by far the most commonly used separation technique for the analysis of TCE metabolites. Most such procedures, including EPA Methods 552[21] and 552.2[22], involve liquid-liquid extraction from water or biological matrices into an organic phase, followed by derivatization and analysis of methyl esters by GC.[21-26] Traditional static headspace methods have been developed by a number of investigators for the analysis of TCA and TCOH in biological samples.[27,28] However, decarboxylation of TCA to chloroform often occurred at high sampling temperatures, and relatively high LODs (limit of detection) were obtained, because no sample pre-concentration steps were involved. A dynamic headspace method for the determination of TCA in urine was developed with a LOQ of 10 $\mu g \cdot L^{-1}$, but simultaneous determination of DCA and TCOH was not achieved.[29] These methods involve the use of solvents and significant numbers of sample preparation steps that are quite time consuming. Solid phase

microextraction (SPME), developed by Pawliszyn et al.[30] was an innovative solvent-free technology. With this method, a coated fiber is exposed to the sample or its headspace and the target analytes partition from the sample matrix into the coating. The fiber bearing the concentrated analyte is transferred to the analytical instrument where desorption, separation, and quantification of the extracted analyte takes place.[31,32] SPME integrates sampling, extraction, concentration and sample introduction into a single step and is therefore an economical and easily automated technique. SPME has been successfully used for the analysis of haloacetic acids, including TCA and DCA, in water, blood or urine.[33-35] Dehon et al[36] reported a SPME GC-ECD method for analyzing TCE, TCA and TCOH in a human fatality. Relatively large sample volumes were used and one of TCE metabolite, DCA, was not analyzed. To our knowledge, no SPME method has been reported for the simultaneous analysis of the TCE metabolites TCA, DCA and TCOH.

A derivatization step is necessary for GC analysis of TCA and DCA due to their low volatility and high polarity. Derivatization reagents such as diazomethane[37], dimethyl sulfate[38], 3-methyl-1-tolytriazene[25], BF_3-MeOH[26] and acidic methanol[24, 27, 39] are often applied to acidic analytes. A mixture of sulfuric acid and methanol is one of the most common derivatizing agents, and no poisonous or explosive chemicals are involved. One study, however, found that using sulfuric acid as a derivatizing reagent resulted in conversion of TCA to DCA.[38]

Negative chemical ionization (NCI) mass spectrometry has increased selectivity and sensitivity for analysis of compounds with electrophilic atoms (halogens), and adds molecular weight specific information.[40,41] The sensitivity of methods using NCI is often increased 3-100 times over electron capture detection (ECD) methods[42], and 2-100 times over electron ionization (EI) mass spectrometry techniques.[43-45] Jia et al[45] developed a GC-NCI-MS method using

pentafluorobenzyl bromide (PFBBr) as a derivatizing reagent for the determination of trace levels of nine haloacetic acids, including TCA and DCA, in plasma, urine, and water with a low LOQ of 25 ng/L.

In the present report, a new method for the analysis for TCA, DCA and TCOH in rat blood using SPME-GC/NCIMS is described. Ethylation rather than methylation of TCA and DCA was chosen to form volatile esters with higher partition constants (the ratio of concentrations of an analyte between the fiber and the headspace) to the fiber.[34] Optimization of the derivatization system and reaction conditions was performed to prevent decarboxylation of TCA and subsequent conversion of TCA to DCA. Headspace SPME parameters were optimized to achieve maximum GC-MS sensitivity. The method was validated and applied to the measurement of concentrations of these metabolites in blood after low-level exposure to TCE.

EXPERIMENTAL

Chemicals and reagents

Analytical grade trichloroethylene (TCE), trichloroacetic acid (TCA), dichloroacetic acid (DCA), 2,2,2-trichloroethanol (TCOH), ethyl trichloroacetate and ethyl dichloroacetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagent grade 2,3-dichloropropionic acid, used as an internal standard, was purchased from Supelco (Bellefonte, PA, USA). Ethyl-2,3-dichloropropionate was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Sulfuric acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC/Spectrophotometric grade ethanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water used in experiments was generated by a Continental deionized water system (Natick, MA, USA). Ultra high purity (UHP) helium and methane were purchased from

National Welders (Charlotte, NC, USA). Alkamuls®, the emulsifying agent used in preparing TCE doses for the animal study, was a generous gift from Rhone-Poulenc (Cranbury, NJ, USA).

Gas chromatography-mass spectrometry

The analyses were carried out on an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass selective detector (Palo Alto, CA, USA). The gas chromatograph was equipped with a 0.75 mm i.d. SPME liner. Separation of the analytes was obtained on a ZB-5MS column (Phenomenex, Torrance, CA, USA: 30m×0.25mm i.d., 0.25 μm film thickness) using helium as the carrier gas (flow rate, 1 mL/min). The gas chromatograph injection port and interface transfer line were maintained at 200 and 280 ºC, respectively. During the fiber desorption process, the injector was operated in the splitless mode. After 2.5 min, the split vent valve was opened to sweep any residual vapors from the liner. The oven temperature was initially held at 35 ºC for 3 min, then increased to 95 ºC at 10 ºC/min, and held for 2 min. The oven was then increased to 280 ºC at a rate of 50 ºC /min and was held for another 3 min. The total run time of a sample was 17.7 min. The mass selective detector was turned off at 13.0 min. The mass spectrometer was operated in negative chemical ionization (NCI) mode with an electron energy of 235 eV. Ultra high purity (UHP) methane was used as the reagent gas at a flow rate of 2 mL/min. The ion source temperature was set at 150 ºC. Selected-ion monitoring (SIM) of m/z 35 and 37 was performed for quantitation of ethyl trichloroacetate, ethyl dichloroacetate, TCOH and ethyl-2,3-dichloropropionate (I.S.). A solvent delay of 2.0 min was set to protect the filament from oxidation.

Preparation of working standard and quality control (QC) solutions

Individual stock solutions of TCA, DCA, TCOH and I.S. (2,3-dichloropropionic acid) were prepared by dissolving 100 mg of the analytes in deionized water to obtain final

concentrations of 10.0 mg/mL and stored at 4 ºC. Combined standard solutions for the calibration curves were prepared from the stock solutions at the following concentrations: 3.33, 8.33, 16.67, 33.33, 83.33, 166.7, 333.3, 833.3, 1666.7 and 3333.3 ng/mL. Standards used to assess precision and accuracy were prepared in deionized water from the stock solutions in concentrations of 3.33, 25, 250 and 2500 ng/mL. A 1.0 μg/mL I.S. standard solution was prepared with deionized water from the 10.0 mg/mL I.S. stock solution. All stock solutions were refrigerated at 4 ºC when not in use and replaced every month. Fresh standard solutions were prepared for each day of analysis or validation.

Preparation of calibration and QC samples

Samples for the calibration curves and QCs were prepared by adding 30 μL of the standard to 100 μL blank blood. This yielded calibration standard concentrations of 1, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng/mL. The final concentrations of QCs were 1, 7.5, 75 and 750 ng/mL. To each 100 μL rat blood sample, 10 μL of the internal standard (1.0 μg/mL, 2,3-dichloropropionic acid) was added.

Sample preparation and in situ derivatization-headspace SPME procedure

In situ derivatization/HS-SPME was optimized using different derivatization reagents such as methanol and ethanol, in order to obtain the methyl and ethyl esters of TCA, DCA and I.S. prior to analysis by HS-SPME. Briefly, 100 μL blood samples were transferred to 2 mL vials containing 400 μL of sulfuric acid (5 mol/L). After addition of 100 μL of ethanol (derivatization reagent), the autosampler vials were quickly sealed with PTFE-coated silicone septa and aluminum caps. The vials were vortexed for 5 min and then heated at 90 ºC for 45 min. After the vials were cooled to room temperature, the vials were placed into the autosampler for analysis. Headspace SPME sampling was performed using a 100 μm polydimethylsiloxane (PDMS) fiber

(Sulpelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland). Fibers were conditioned at 250 ºC for 30 min prior to use. Sample vials were preheated in the agitator with an agitator speed of 500 rpm for 30 min before analysis. The SPME fiber was then exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 10 min at 30 ºC under agitation, the fiber was withdrawn into the needle and immediately desorbed at 200 ºC for 1 min into the GC injection port. Several parameters affecting derivatization and HS-SPME were studied: derivatization reagent (methanol or ethanol), concentration of sulfuric acid (catalyst, 0.2-5 mol/L) and volume of derivatization reagent (20-200 μL). Other parameters affecting the HS-SPME procedure, such as sample preheating time (up to 60 min), derivatization-extraction time (3-25 min), derivatization-extraction temperature (30-90 ºC), desorption temperature (150, 200 and 250 ºC), desorption time (60-150 sec), agitator speed (250, 500 and 750 rpm) and fiber positions in sample vials and GC injector were also optimized. Possible carryover was prevented by heating the fiber for 3 min at 250 ºC between samples. Two blanks were run before each batch of samples to confirm the absence of contaminants. For optimization, all determinations were performed in duplicate, and the average values reported.

Method validation

The methods were validated for linearity, recovery, accuracy and precision. Blood calibration curves were generated by linear regression analyses of the peak area ratios of TCA, DCA or TCOH to that of the I.S. versus the concentration of each analyte applying a weighting scheme of $1/x^2$. Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated using four QC samples. Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated 3

times over 3 days in order to determine the inter-day accuracy and precision (n=15). Relative recoveries were calculated for spiked samples at 1, 7.5, 75 and 750 ng/mL (n=5) by dividing the peak areas for analytes spiked in rat blood by the peak area for an equal concentration of analyte in deionized water (n=5). The stability of the stock solutions was determined at their storage conditions of 4 ºC for one month. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls.[46] The bench-top stability of spiked blood samples stored at room temperature was evaluated for 8 hr. The autosampler stability was evaluated over a period of 24 hr to determine if there was any loss of signal due to the time a sample spent in the autosampler. The refrigerator stability was investigated by comparing stability of samples following storage in the refrigerator at 4 ºC over a period of 5 days against freshly spiked samples. The stability testing was performed at 7.5 and 750 ng/mL concentration levels for all of the analytes.

Sampling

Male Sprague-Dawley (S-D) rats were obtained from Charles River Laboratories (Raleigh, NC, USA). The rats were acclimated (2 rats/cage) for at least 7 days in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) -approved animal care facility after arrival. All experiment protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia. The rats were cannulated so serial blood samples could be taken to characterize the time-course of TCE and TCE metabolites. Briefly, each rat was anesthetized and a cannula was inserted into the right carotid artery. Water was provided *ad libitum*, but food was withheld during the 24 hr recovery period following cannulation before dosing. Food was provided 3 hr after dosing. TCE was given orally at a concentration of 1 mg/kg using 5% Alkamulus as the vehicle. Blood samples from each rat (n=4)

were taken 2, 5, 10, 15, 30, 60, 120, 240, 360 and 480 min post dosing. Blood samples were analyzed immediately for TCE determination using a method developed previously.[47] The samples to be analyzed for TCE metabolites were stored at 4 ºC until they could be analyzed (within 5 days).

RESULTS AND DISCUSSION

Optimization of the derivatization system

Optimization of the derivatization system including selection of solvents for the standards, derivatization reagent (methanol versus ethanol), volume of derivatization agent, and concentrations of sulfuric acid were performed, in order to maximize the reaction yields for the TCA and DCA esters. For optimization experiments, the HS-SPME extraction temperature and time were maintained at 50 ºC and 10 min, respectively. The preheating time for the samples was kept at 30 min. These preheating and extraction times were considered to be long enough, based on the following optimization experiments described below for SPME parameters. A commercially available 100-μm PDMS fiber was selected, taking into account its utility in a previous study, in which it was utilized to extract ethyl haloacetates from water by headspace SPME.[34]

Solvents for the standards.

Several solvents for the standards were tested including acetonitrile, methanol, ethanol and deionized water. In each case, the organic solvents completely dissolved the analytes. Interestingly, responses were found to be 10-100 times lower when organic solvents were used rather than deionized water. TCA, DCA and TCOH are polar compounds, so that they may not distribute well in organic solvents. Therefore, the actual amounts of the analytes extracted by the SPME fiber from the biological matrix (i.e., blood) may be lower than when dissolved in water.

The sensitivity of the HS-SPME method was dependent upon solubilization of the analytes. Deionized water was selected to prepare the standard solutions due to their relatively high solubility in water.

Acidic ethanol esterification versus acidic methanol esterification.

The attributes of methanol and ethanol as derivatizing agents for TCA and DCA were compared. To avoid the conversion of TCA to DCA, each analyte was analyzed individually for methyl esters or ethyl esters over a range of concentrations (10-1000 ng/mL). The responses for the TCA ethyl ester were only 5% higher than those for TCA methyl ester, but the linearity for the ethyl ester (r^2 = 0.992) was better than for the methyl ester (r^2 = 0.979). The DCA ethyl ester showed a 40% increase in peak area and better linearity (0.990 versus 0.963) than did the methyl ester. According to a previous study, volatile ethyl esters of haloacetic acids demonstrated higher SPME fiber partition coefficients than methyl esters.[34] Therefore, acidic ethylation of TCA and DCA was selected for our study.

Derivatization reagent amount.

The concentration of the derivatization reagent could affect both the reaction yield of TCA or DCA ethyl esters and the signal response for TCOH. Amounts of ethanol ranging from 20 to 200 µL were tested (Figure 6.1A). DCA and TCA ethyl ester responses increased with increasing volume of ethanol. Volumes of ethanol between 100 and 200 µL gave satisfactory reaction yields of DCA and TCA. The highest responses for TCA and DCA were obtained at 200 and 100 µL, respectively. TCOH responses, however, decreased with increase in the ethanol volume. This decrease could be due to the similar structures of TCOH and ethanol, resulting in competition for binding sites on the fiber. Considering this signal suppression effect of ethanol

on TCOH, 100 µL of ethanol was selected as a compromise for concurrent analysis of TCA, DCA and TCOH.

In this acidic ethylation system, sulfuric acid acts as a catalyst. Experiments conducted to optimization the sulfuric acid concentration demonstrated that the amount of sulfuric acid affected the derivatization efficiency of DCA and TCA. To avoid the conversion of TCA to DCA, each analyte was tested individually. 400 µL of different concentrations of sulfuric acid, ranging from 0.2 to 5 mol/L, were tested with the ethanol volume fixed at 100 µL (Figure 6.1B). TCOH responses were not affected by the amount of sulfuric acid. Interestingly, at low acid concentrations (0.2 to 1 mol/L), conversion of TCOH to DCA (13%) and TCA (3%) was found. However, this degradation of TCEOH was significantly reduced (less than 0.5% total) at higher sulfuric acid concentrations (2 to 5 mol/L). DCA and TCA ethyl esters demonstrated a dramatic increase in peak area when the concentration was increased from 2 to 5 mol/L. In addition, the higher concentration of sulfuric acid also avoided the conversion of TCA to DCA. The conversion of TCA to DCA was greatly reduced by using the low pH (reduced from≈ 8% to less than 0.2%). It appears that the high concentrations of sulfuric acid to force the equilibrium of TCA even further toward the neutral carboxylic acid form (pKa≈ 0.5). Thus, 5 mol/L of sulfuric acid was chosen for this study.

Optimization of the derivatization-headspace SPME conditions

After optimization of the derivatization process had been accomplished, other conditions, such as derivatization time and derivatization temperature, and the parameters that affect the sensitivity of the headspace SPME method, including extraction temperature and time, desorption time, desorption temperature, fiber positions in the GC injector and SPME vials, and

agitator speed were optimized. The volume of ethanol was fixed at 100 µL, and the concentration of sulfuric acid was fixed at 5 mol/L.

Derivatization-extraction temperature.

The effect of sample temperature on the derivatization/HS-SPME was examined from 30 to 90 ºC (data not shown). In general, initial increases in temperature modestly enhanced the derivatization reaction yield. Moreover, the mass transfer process was also favored by increasing the vapor pressure of the analytes in the headspace. But at high temperatures (above 50 ºC), the affinity of the analytes for the fiber coating diminished, due to a decreased fiber coating/headspace partition coefficient. Thus the signal responses decreased. Therefore, 50 ºC was selected as the optimal temperature for derivatization and SPME extraction for all analytes.

Derivatization-extraction time.

Because there was little difference in TCOH's and DCA's peak area over the temperature range from 30 to 60 ºC, different extraction times (3-25 min) were examined concurrently, in order to determine the optimal extraction conditions for all metabolites. Figure 6.2 shows the effect of extraction temperature and time on peak areas of TCA, DCA and TCOH in rat blood. Comparing the extraction time profiles obtained at different temperatures for TCA (Figure 6.2A) reveals that higher sampling temperatures will increase the speed of equilibrium. At 30 ºC, the equilibrium status could not be reached within 25 min. At high extraction temperatures, a shorter extraction time (15 min at 40 ºC, 10 min at 50 ºC or 5 min at 60 ºC) was required to reach equilibrium. At 60 ºC, decarboxylation of TCA to chloroform occurred, and a minor chloroform peak was found in the chromatogram (0.2%). DCA behaved similarly to TCA. Different extraction times and extraction temperatures did not affect the extraction efficiency of TCOH

significantly. To minimize the analysis time, and to prevent analyte degradation, 10 min at 50 ºC was selected for this technique.

Sample preheating time.

For this *in situ* HS-SPME derivatization process, sample preheating time proved to be an important parameter that affected the derivatization efficiency and extraction equilibrium conditions. The preheating process improved the mass transfer kinetics of the derivatized analytes from the liquid sample to the headspace and shortened the equilibrium time. Following addition of 100 µL of ethanol and 400 µL of 5 mol/L sulfuric acid, vials were preincubated in an agitator for 1, 3, 5, 10, 15, 20, 25, 30, 40, 50 and 60 min and extracted for 10 min at 50 ºC. For TCA and DCA, the highest response was obtained at 30 min. These responses increased more than 50 % between 1 and 30 min. Longer preheating times did not further improve recovery. For TCOH, which did not need to be derivatized, sample preheating time did not affect the sample response. Thus, 30 min was selected as the sample preheating time for this procedure. However, when samples were preheated for 30 min and extracted for 10 min at 50 ºC, the peak areas for the I.S. were not constant. The response for the I.S. increased with time, which caused the validation to fail. However, the peak areas for the internal standard were consistent if samples were preheated at 90 ºC for an additional 45 min. Our hypothesis for this behavior is that the IS has one more carbon atom and is therefore bigger than DCA or TCA. Hence, the equilibrium time for IS in derivatization reaction is expected to be much longer than for DCA or TCA. After we heated the samples at 90 ºC for an additional 45 min, the equilibration of IS's derivatization reaction could be reached making the peak areas for the internal standard consistent.

Agitator speed and fiber positions in SPME vials and GC injector.

The effect of agitator speed was tested over a range from 250 to 750 rpm. The depth of the fiber in the vial and in the GC injector was also examined over the ranges of 22-31 and 45-54 mm, respectively. Optimization results (data not shown) demonstrated that neither agitator speed nor fiber insertion depth influenced the results. Therefore, the default parameters, 500 rpm, 25 mm in SPME vials, 48 mm in GC injector, were selected for this study.

Desorption time and temperature.

Finally, desorption time and temperature were optimized. Three desorption temperatures (150, 200 and 250 ºC) were evaluated for a desorption time of 2 min. Responses for the three analytes at 200 and 250 ºC were higher than at 150 ºC, but there were no significant differences in between 200 and 250 ºC. In addition, carryover was not detected at either temperature. Therefore the lower temperature, 200 ºC, was chosen for further experiments to avoid degradation of the fiber at higher temperatures. At a desorption temperature of 200 ºC, a range of desorption times from 60 to 150 sec was tested. No significant differences were observed among these parameters, and no carryover effects were found, even following analysis of blood samples spiked with the highest amounts of analyte. Therefore, the shortest desorption time, 60 seconds, was selected for this study to extend the life of the fiber.

Validation of the method

The performance characteristics of the *in situ* derivatization/HS-SPME GC/MS method were evaluated with respect to specificity, linearity, precision， accuracy, limits of quantitation, recovery and stability.

Identification of metabolites in Negative Chemical Ionization mode.

In the negative chemical ionization mode, no molecular peaks for TCA, DCA, TCOH or the I.S. were found. Instead, the primary fragment ions, ^{35}Cl and ^{37}Cl, were the base peaks in each mass spectrum. In NCI mode, thermal electrons are first created from a heated metal filament. When neutral molecules interact with these thermal electrons, electron capture occurs to generate $M^{-\bullet}$ ions. As $M^{-\bullet}$ is an odd-electron species, homolytic bond cleavages as well as rearrangement fragmentations may occur. Therefore, the major fragment ion of TCA and DCA, $Cl^{-\bullet}$, was present in the mass spectra. Quantitation of these TCE metabolites, using chlorine ions in the NCI mode, decreased some of the selectivity of mass spectrometry. Determination of these different chlorine-containing species was based solely on GC retention times and the presence of chlorine. The GC retention time for each analyte and the I.S. was verified by its EI mass spectrum. Separation of the parent compound TCE and its metabolites were also investigated to insure the specificity of the method. All of these analytes, including TCE, TCA, DCA, TCOH and the I.S., were well separated. Representative chromatograms obtained from blank blood and blood spiked with the LLOQ standard (1 ng/mL) are shown in Figure 6.3. No other interfering peaks from endogenous compounds were observed at the retention times of the analytes or the I.S. in blank rat blood except TCA. TCA is one of the most frequently reported chlorination byproducts in tap water. Blank blood from rats that were exposed to TCA contaminated water were found to have a low, background level of TCA (a small peak at Rt = 9.15 min).

Linearity and LLOQ.

The calibration curves generated for the validation of the three TCE metabolites showed a good linear response (R^2>0.994) over the range from 1 to 1000 ng/mL. SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-

weighting scheme was used for generating calibration curves. The lower limit of quantitation, LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 1 ng/mL for DCA and TCOH in blood. A signal-to-noise (S/N) ratio > 10 at the LLOQ was observed for both analytes in rat blood. Due to the presence of TCA in the blank rat blood (0.09 – 0.12 ng/mL), its LLOQ was defined as the concentration at which the peak intensity was 10 times higher than the background with the same 20% limitations for precision and accuracy. One ng/mL for TCA was determined to be as the LLOQ, although its sensitivity was much higher than for DCA and TCOH at the same concentration. To our knowledge, this is the first SPME GC-NCI/MS method for the determination of the three major TCE metabolites in blood. It is the first time that the LLOQ for TCOH in blood has been validated as low as 1 ng/mL.

Precision and accuracy.

Assay precision and accuracy for TCA, DCA and TCOH in rat blood were established at the LLOQ and at low, medium, and high concentrations over 3 days. Table 6.1 summarizes these accuracy and precision data that were collected. The intra-day precision and accuracy (n=5) were better than 5.91% and 7.84% for all the analytes. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were lower than 7.74% and 7.34%, respectively.

Relative recoveries.

The relative recoveries for three analytes were calculated by comparing the amount extracted from rat blood by the HS-SPME method with the amount extracted from water. Samples were prepared in four concentrations: 1, 7.5, 75, and 750 ng/mL. For each concentration, 10 samples were prepared, 5 in rat blood and 5 in deionized water. Following the

same *in situ* derivatization HS-SPME process, relative recoveries were determined by comparing the peak area for each analyte in blood and in water (Table 6.2). Relative recoveries of all analytes and the I.S. ranged from 84.1% to 100.9%. Recoveries were lowest for the two highest TCA concentrations. Nevertheless, the efficiency of the SPME extraction in blood and water was quite comparable.

Stability studies.

Stability testing is very important for validated methods for analysis of biological samples. Our stock solutions were stable at the selected storage condition (4 ºC) for one month (data not shown). All the other stability studies were conducted at two concentrations of each TCE metabolite (7.5 and 750 ng/mL), with five determinations for each. The total derivatization and HS-SPME extraction time for each sample is about 40 min. During the processes of validation and analysis, samples may sit in the autosampler for hours. Therefore, examining the loss or degradation of the analytes during this time is critical. Blank blood samples were spiked with all analytes and left in the autosampler at room temperature for 24 hr. These samples were then compared with samples prepared freshly. The obtained values for precision were less than 8.56%, and the relative error was less than 12.32% (see Table 6.3). The bench-top stability results indicated that spiked samples were stable for all of the analytes for 8 hr. The relative error ranged from 0.84% to 10.27% (< 15%), and the RSD varied from 1.03% to 7.85% for all the analytes (Table 6.3). Because freezing blood lyses the blood cells, resulting in different relative recoveries, the stability of refrigerated blood samples was evaluated. Blood samples were spiked with 7.5 and 750 ng/mL of each analyte, and these samples stored at 4 ºC. Five days later, they were compared with prepared freshly samples. Values obtained for precision and relative error were less than 13.90% and 12.92%, respectively. According to criteria for method validation,

analytes were considered stable if the relative errors (% RE) of the mean test responses were within 15% of the appropriate controls.[46]

Applications

This *in situ* derivatization HS-SPME GC/MS method was applied to study the toxicokinetic behavior of TCE and its metabolites in rodents given a low oral TCE dose. Sprague-Dawley rats (n=4) were gavaged with 1 mg/kg of TCE as an aqueous emulsion. Serial blood samples were collected and analyzed immediately for TCE using a previously developed method[47]. Blood samples were then assayed for TCE metabolites using the presently-reported derivatization SPME-GC/MS method. Figure 6.3 (c) shows a chromatogram of TCE's metabolites (TCA, DCA and TCOH) in blood taken 2 min after a rat was dosed orally with 1 mg/kg of TCE. Blood TCE, TCA, DCA and TCOH concentration versus time profiles were obtained and shown in Figure 6.4. TCE was absorbed quickly from the GI tract and reached a maximum concentration in the blood at 5 min. TCE was efficiently distributed to body tissues, resulting in a rapid decrease in its concentrations in the blood. The profile for TCOH was similar to that for TCE. The highest concentrations of TCOH were observed 10-15 min post dosing. TCOH levels in the blood diminish rapidly thereafter. In contrast, TCA concentrations in the blood progressively increased and reached a maximum concentration of 280 ng/mL after 1 hr. TCA levels remained nearly constant over the next 8 hr. Half-lives of 9-10 hours were reported for rats after administration of 5 mg/kg TCA by intravenous infusion.[48] Its long half-life is attributable to poor metabolism and high plasma protein binding. DCA concentrations in blood fluctuated between 3 and 7 ng/mL from the beginning to the end of the monitoring period. Low levels were expected, since DCA is formed from other TCE metabolites and is a more minor metabolite in rats and humans.

CONCLUSIONS

In this paper a specific, rapid and sensitive derivatization HS-SPME GC-NCIMS method for the determination of TCE metabolites, i.e. TCA, DCA and TCOH, in rat blood has been developed and validated. With acidic ethylation, TCA and DCA were simply derivatized and then extracted by SPME PDMS fibers. In comparing with other derivatization reagents, acidic ethanol was simpler, safer and more efficient. This validated method yielded excellent linearity, precision and accuracy over 3 orders of magnitude and only required small sample volumes. The specificity of this method lies in the presence of more Cl atoms in the molecules, allowing the use of NCI as an optimal ionization method that greatly improves the signal-to-noise ratio of the analytes in complex biological matrices. The limit of quantition for the three TCE metabolites was 1 ng/mL of blood. To our knowledge, this is the first validated derivatization headspace SPME-GC/NCIMS method for determination of the major TCE metabolites, TCA, DCA and TCOH, in blood. This method was successfully applied to study the toxicokinetic behavior of TCE metabolites following oral administration of a 1 mg/kg oral dose of TCE. The method should be suitable for toxicokinetic and metabolic studies of TCE.

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Table 6.1. The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the HS-SPME-GC/MS method used to quantitate TCE metabolites in rat blood

Concentration added (ng/mL)	Intra-day			Inter-day		
	Observed Concentration ±S.D. (ng/mL)	R.S.D. (%)	Error (%)	Observed Concentration ±S.D. (ng/mL)	R.S.D. (%)	Error (%)
TCOH						
1	0.988±0.048	4.83	4.38	1.031±0.053	5.10	5.14
7.5	7.446±0.227	3.05	1.98	7.530±0.583	7.74	6.22
75	75.16±2.861	3.81	3.20	75.63±2.687	3.55	3.34
750	735.9±9.441	1.28	1.88	774.4±34.66	4.48	4.50
DCA						
1	1.034±0.061	5.91	5.95	1.056±0.079	7.46	7.34
7.5	7.075±0.239	3.38	5.67	7.423±0.498	6.71	5.60
75	70.14±1.384	1.97	6.48	71.19±2.741	3.85	5.58
750	691.2±19.02	2.75	7.84	728.03±39.08	5.37	5.17
TCA						
1	1.056±0.041	3.89	5.58	1.025±0.076	7.44	6.65
7.5	7.521±0.255	3.39	2.73	7.786±0.497	6.38	5.53
75	73.47±1.370	1.87	2.37	74.15±2.558	3.45	2.69
750	748.8±9.962	1.33	0.86	769.7±29.16	3.79	3.03

Table 6.2. The relative recoveries (%) (mean ± S.D.) of TCE metabolites and I.S. in rat blood (n=5)

Concentration (ng/mL)	TCOH	DCA	TCA	I.S.
1	95.5±8.15	93.5±5.39	92.8±6.27	
7.5	94.0±2.39	98.9±3.32	93.5±8.10	
75	96.7±5.25	99.1±3.89	88.7±4.84	
750	99.3±1.94	97.5±1.19	84.1±2.44	
100				100.9±6.17

Table 6.3. Stability testing of TCE metabolites in rat blood, (n=5)

Compounds	Stability	Spiked conc. (ng/mL)	Observed conc. ±SD (ng/mL)	R.S.D. (%)	Relative error (%)
TCOH	Refrigerator (5 day)	7.5	8.47±1.12	13.2	12.92
		750	761.4±9.50	1.25	1.52
	Bench-Top (8 hr)	7.5	7.21±0.44	6.09	-3.88
		750	760.1±18.11	2.38	1.35
	Autosampler (24 hr)	7.5	8.42±0.32	3.84	12.32
		750	804.1±25.33	3.15	7.21
DCA	Refrigerator (5 day)	7.5	7.38±1.03	13.9	-1.61
		750	738.8±30.92	4.18	-1.49
	Bench-Top (8 hr)	7.5	6.73±0.53	7.85	-10.27
		750	743.7±7.67	1.03	-0.84
	Autosampler (24 hr)	7.5	7.26±0.61	8.39	-3.22
		750	661.6±9.36	1.42	-11.78
TCA	Refrigerator (5 day)	7.5	7.63±0.44	5.78	1.74
		750	692.6±25.86	3.73	-7.65
	Bench-Top (8 hr)	7.5	7.97±0.59	7.37	6.26
		750	710.4±17.42	2.45	-5.28
	Autosampler (24 hr)	7.5	7.22±0.62	8.56	-3.82
		750	682.8±22.24	3.26	-8.96

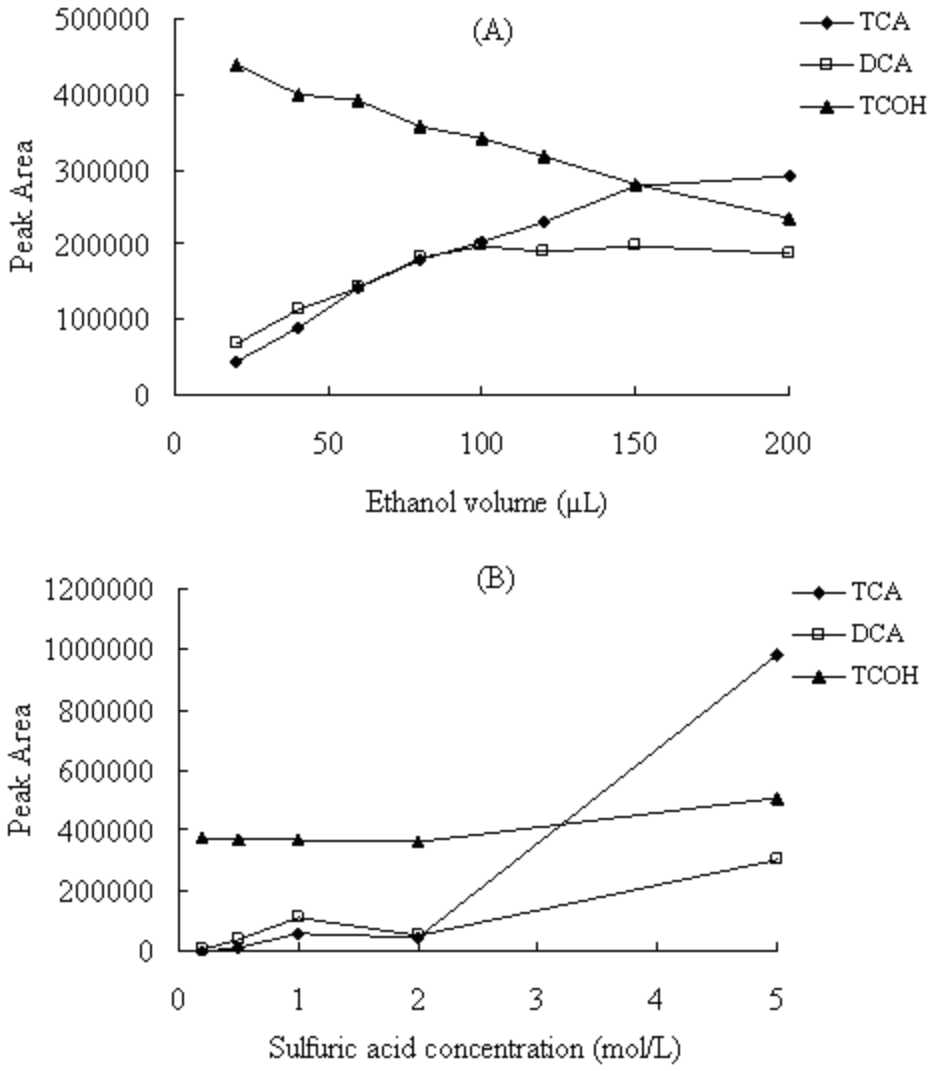

Figure 6.1. Effect of volume of ethanol (A) and concentration of sulfuric acid (B) on peak areas of TCA, DCA and TCOH. HS-SPME conditions: 50 ng of each analyte were derivatized with ethanol and sulfuric acid; 100-µm PDMS fiber; extraction temperature, 50 ºC; extraction time, 10 min; preheat time, 30 min. (A) 400 µL of 1 mol/L sulfuric acid; (B) 100 µL ethanol.

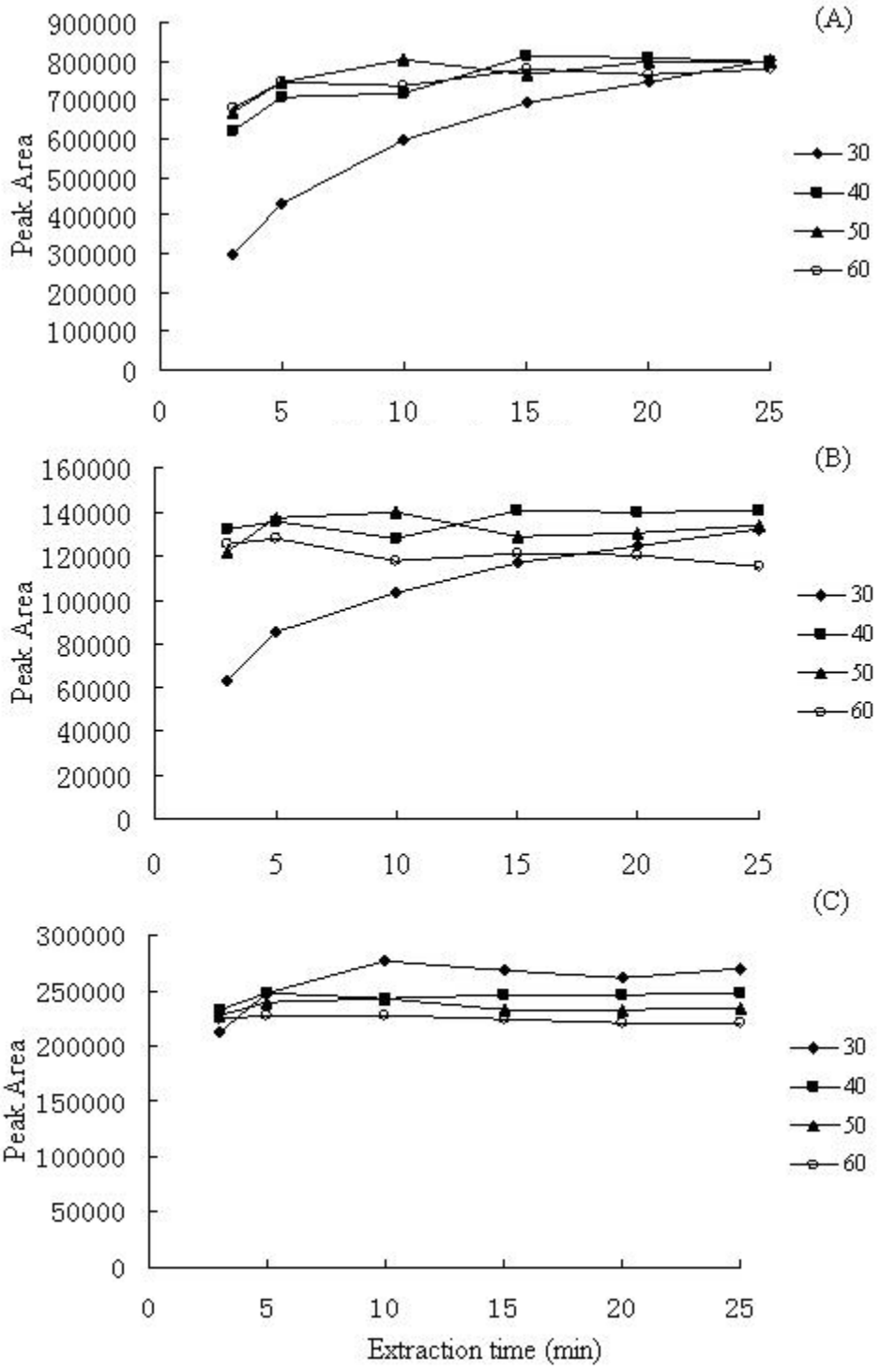

Figure 6.2. Plot of (A) TCA ethyl ester; (B) DCA ethyl ester; (C) TCOH peak area versus extraction time obtained at different extraction temperatures (30, 40, 50 and 60 ºC). HS-SPME conditions: 50 ng of each analyte was derivatized with 100 µL ethanol by addition of 400 µL of 5 mol/L sulfuric acid; 100-µm PDMS fiber; extraction time, 10 min; preheat time, 10 min.

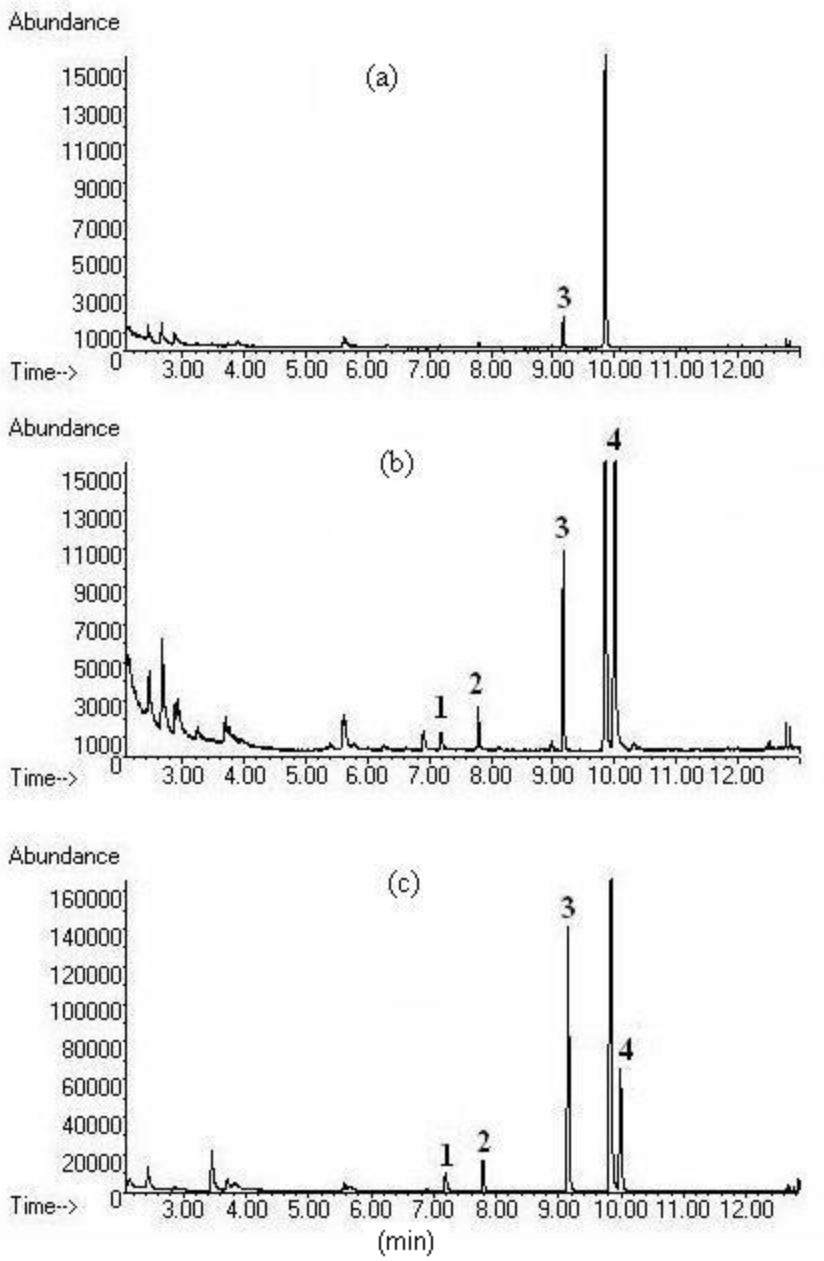

Figure 6.3. Representative chromatograms obtained from (a) blank blood; (b) blood spiked with the LLOQ (1 ng/mL) concentration of TCE metabolites; (c) blood sample taken after 2 minutes from a rat dosed with 1 mg/kg TCE orally. Peak identifications: 1 = TCOH; 2 = DCA; 3 = TCA; 4 = 2,3-Dichloropropionic Acid, I.S.

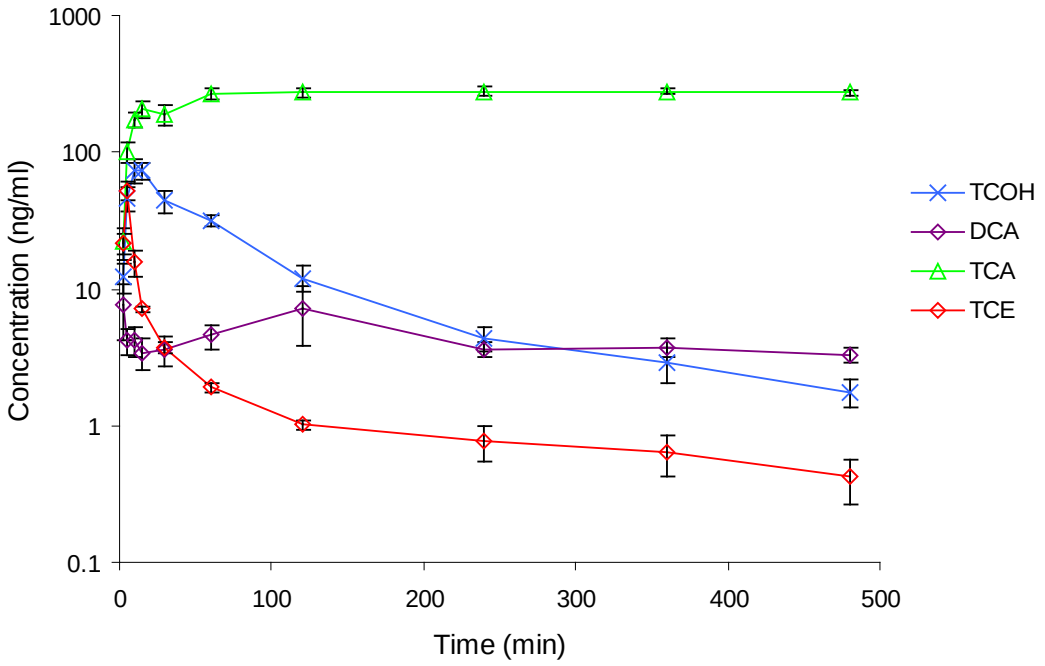

Figure 6.4. Blood concentration versus time profiles of TCE, TCA, DCA and TCOH from SD rats dosed orally with 1 mg/kg of TCE (mean concentration ± standard error of the mean (SEM), n=4 for each time-point)

CHAPTER 7

CHARACTERIZATION OF PRESYSTEMIC ELIMINATION OF TRICHLOROETHYLENE (TCE) IN RATS FOLLOWING ENVIRONMENTALLY-RELEVANT EXPOSURES

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ABSTRACT

TCE is volatile organic chemical (VOC) that has been used extensively as a degreaser, solvent and dry cleaning agent. As a result of its widespread use, TCE is the most common contaminant in groundwater at U.S. hazardous waste sites. Human exposure to TCE is of concern, because high, chronic doses are carcinogenic in mice and rats. In order to characterize the toxicokinetics (TK) of TCE following ingestion of environmentally-relevant (i.e., trace) doses, a valid, sensitive and rapid analytical method was developed using headspace solid-phase microextraction (HS-SPME) and gas chromatography with negative chemical ionization (NCI) mass spectrometry (GC-NCIMS). The lower limit of quantitation was 0.025 ng/ml from blood. The right carotid artery of male Sprague-Dawley rats was cannulated. TCE was given by oral bolus or iv, in doses of 0.001, 0.01, 0.1, 1.0, 2.5, and 10.0 mg/kg bw in an aqueous Alkamuls® emulsion. Serial 200-µL blood samples from each rat were taken and analyzed immediately to obtain concentration-time profiles. WinNonlin software was used to determine TK parameters. TCE concentrations were measurable at the 0.001 mg/kg dose for only the first 15 min post dosing. At 0.01 mg/kg, TCE was detectable for 120 min. Thus the concentrations in the terminal elimination phase were not above the LOQ. TK parameters could be ascertained for the 0.1-10.0 mg/kg dosage range. Bioavailability ranged from 12.5 to 25.5 %, and was about 2-fold higher for the 10 mg/kg oral doses that for the lower doses. Clearance ranged from 49.7 to 68.8 ml/min·kg and was constant in this dosage range. A miniscule amount of TCE (AUC_0^{15} = 0.9 ng·min/ml) reached the systemic circulation of rats ingesting 0.001 mg/kg. This dosage would be received by a 70 kg person who consumed 2 L water containing 35 ppb TCE. Our analytical method makes it possible to directly assess the efficiency of presystemic elimination of trace levels of TCE.

INTRODUCTION

Trichloroethylene (TCE) is a halogenated volatile organic compound (VOC) that has been used extensively as a metal degreaser, chemical intermediate, anesthetic and drying cleaning agent. Much of the TCE used in the United States is released into the atmosphere from vapor degreasing operations. TCE contamination of ground and surface waters is largely attributed to industrial discharge of the chemical and leaching from hazardous waste sites (Bakke *et al.*, 2007; ATSDR, 1997; Wu and Schaum, 2000). As a result of its widespread use, TCE can be found in the groundwater in the proximity of more than 50% of the hazardous waste sites on the United States Environmental Protection Agency's (EPA) National Priorities List (Fay and Mumtaz, 1996). According to a survey by the National Water-Quality Assessment (NAWQA) program, 5% of wells throughout the United States have detectable levels of TCE, with concentrations ranging from 0.02 ug/L to 230 ug/L (Moran *et al.*, 2007). Extremely high, chronic doses of TCE were required to produce tumors in mice and rats (Bull, 2000; Rhomberg, 2000; EPA, 2001). Nevertheless, exposure of humans to very low levels of TCE is of concern, primarily because of the potential carcinogenic risks.

Chemical toxicity is dependent upon a number of dynamic processes including systemic absorption, distribution, metabolism, and elimination of a chemical from target tissues and the remainder of the body. Gaining an understanding of these processes is helpful in interpreting toxic effects of the chemical under different exposure conditions. It is generally recognized that drugs and chemicals absorbed from the gastrointestinal tract must first pass through the GI wall and then from the portal blood into the liver and onto the lungs, before entering the arterial circulation and distributing to tissues throughout the body. This phenomenon is referred to as first-pass or presystemic elimination (Routledge and Shand, 1979; Gibaldi and Perrier, 1982).

The remainder of the body may thus be afforded protection against exogenous chemicals. First-pass effect has been studied for a number of drugs, in order to estimate systemic bioavailability (*F*) (Yang and Lee, 2008; Hanada *et al.*, 2008; Lee and Lee, 2007; Yan *et al.*, 2007; Hao *et al.*, 2005; Martin *et al.*, 2003). There is little definitive information available on first-pass elimination of TCE and some other halocarbons. Presystemic elimination plays an important role in the disposition of TCE following oral exposure. It has been theorized that low oral doses of VOCs are completely removed by presystemic or first-pass elimination (Anderson, 1981). One previous study of TCE demonstrated that significant proportions of low oral given doses of TCE were removed by the liver and lungs (Lee *et al.*, 1996). The presystemic elimination of TCE was inversely related to the dose with as much as 60% of the lowest doses removed. However, it has not been possible to directly measure the efficiency of presystemic elimination of environmentally-relevant exposures of TCE due to the lack of an analytical method with adequate sensitivity.

Toxicokinetic studies have shown that TCE is rapidly and extensively absorbed from the GI tract (Lash *et al.*, 2002). Once present in the systemic circulation it is efficiently metabolized. Some of doses of TCE are eliminated via the lungs, while more than 70% is biotransformed to its major metabolites, trichloroethanol and trichloroacetic acid (Lash *et al.*, 2002; Goeptar *et al.*, 1995). The toxicokinetic behavior of TCE is not linear over a wide range of doses due to saturated metabolism. This capacity-limited route of elimination can be expressed by Michaelis–Menten kinetics (Gibaldi and Perrier, 1982), in which the rate of elimination does not always increase in proportion to the blood concentration. At low and intermediate doses of a chemical with a high hepatic extraction ratio, such as TCE, the limiting factor in the rate of elimination is the blood flow to the liver, so that first-order kinetics is obeyed. As dose increases, the capacity

of metabolizing enzymes can be exceeded, and the kinetics shift from first-order to zero-order (Andersen *et al.*, 1980; Andersen, 1981). Therefore, hepatic clearance is no longer constant and first-pass elimination decreases. In fact, saturation of first-pass metabolism has been observed at lower doses as compared to saturation of hepatic clearance. Previous studies indicated that TCE was eliminated through dose-dependent nonlinear processes (Lee *et al.*, 1996; 2000). A range of doses adequate to the transition of nonsaturated to saturated elimination were administered in their studies. When the oral dose exceeded 8 mg/kg, the key isoenzyme (P4502E1) metabolizing TCE was saturated and nonlinear kinetics of TCE was observed. However, the lower oral doses were not included in their studies. There have been some *in vivo* studies involving serial measurements of a halocarbon in blood, in which changes in pharmacokinetic parameters have been delineated (D'Souza *et al.*, 1985; Angelo *et al.*, 1986). All of these were based on the data acquired from saturated doses in rats. A kinetics study, of a range of doses lower than the saturated dose and close to the environmental exposures, is required to characterize properly the elimination of TCE. The findings from the low dose study would be more important to human risk assessment of TCE exposures.

In this study, a valid, sensitive and rapid headspace solid-phase microextraction (HS-SPME) and gas chromatography with negative chemical ionization mass spectrometry (GC-NCIMS) method and a HS-SPME GC with electron ionization (EI)-MS method were used for the analysis of TCE samples. The lower limit of quantitation was 0.025 ng/ml for the NCI method and 0.25 ng/ml for the EI method. The specific aims of the current study were: (1) to characterize the toxicokinetics (TK) of TCE following ingestion of environmentally-relevant (i.e., trace) doses in rats, and (2) to assess the efficiency of first-pass elimination of oral TCE in rats.

MATERIALS AND METHODS

Chemicals. Analytical grade trichloroethylene (TCE) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfuric acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, USA). Deionized water used in experiments was generated from a Continental deionized water system (Natick, MA, USA). Ultra high purity (UHP) helium and methane were purchased from National Welders (Charlotte, NC, USA). Alkamuls, a polyethoxylated vegetable oil, was obtained from Rhone-Poulenc (Cranbury, NJ, USA) and used in a concentration of 5% (v/v) in 0.9% saline to prepare stable aqueous suspensions for dosing the animals.

Animals. Male Sprague-Dawley (S-D) rats (270-380 g) were obtained from Charles River Laboratories (Raleigh, NC, USA). All experiment protocols for this study were approved by the Institutional Animal Care and Use Committee at the University of Georgia. The animals were housed in pairs in polycarbonate cages in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) -approved animal care facility with a 12-h light/dark cycle (light: 7:00 A.M.-7:00 P.M.) at 22 ± 2 ºC and 55 ± 5 % relative humidity for at least 7 days prior to use. Food (5001 Rodent Diet, PMI Nutrition International, Brentwood, MO, USA) and boiled tap water were provided *ad libitum* during this period. Each rat was anesthetized 18-24 h prior to TCE dosing by IM injection of 0.075-0.1 ml/100 g bw of a “cocktail” composed of ketamine HCl (100 mg/ml), acepromazine maleate (10 mg/ml), and xylazine HCl (20 mg/ml) (3:2:1, v/v/v). A cannula (PE-50, polyethylene tubing) was surgically inserted into the carotid artery and jugular vein. The jugular vein cannula was used for IV injection. Serial blood samples were taken from the carotid artery for both IV and oral studies. The cannulas were filled with heparinized saline, passed under the skin, and exteriorized at the nape of the neck in order to

maintain patency and prevent the freely moving animals from disturbing them. Boiled water was provided *ad libitum*, but food was withheld during the 24-h recovery period, in order to avoid interanimal variability in GI absorption due to varying food intake. Access to food was provided 1 h after TCE dosing.

Dosing. Five to seven rats were assigned to each group. TCE was incorporated into a 5% aqueous Alkamuls emulsion the day of the experiment and injected over 30 seconds into jugular vein or given orally (PO) to the unanesthetized animals. Doses of TCE employed for IV studies were 0.1, 1 and 2.5 mg TCE/kg bw. One higher dose (5 mg/kg) and three lower doses (0.01, 0.001 and 0.0001 mg/kg) were also given orally to the rats. It was not advisable to exceed 5 mg/kg, due to the high sensitivity of the analytical method. High concentrations of TCE exceed the high limit of quantitation (100 ng/ml) of the analytical method and would saturate the mass spectrometry detector.

Blood collection and TCE analysis. Every time before dosing, blank blood samples were collected from each rat and analyzed to insure there was not a detectable background level of TCE before beginning. Serial blood samples were taken from the carotid artery cannula at 1- to 480- minute intervals post dosing. One milliliter of 0.9% saline was infused ia 1 h postdosing to replace the blood taken during the serial sampling.

TCE concentrations in blood were analyzed by the Headspace Solid-phase Microextraction (HS-SPME) Gas Chromatography Mass Spectrometry (GC-MS) method of Liu *et al.* (2008a; 2008b). Two hundred microliters of whole blood were immediately transferred into SPME autosampler vials containing 400 µl of sulfuric acid (1 mol/L) and 20 µl of acetonitrile. All the autosampler vials were quickly sealed with PTFE-coated silicone septa and crimped aluminum caps. The vials were vortexed for 3 min and set in the autosampler for analysis.

Headspace SPME sampling was performed using a 100-μm polydimethylsiloxane (PDMS) fiber (Sulpelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland). Fibers were conditioned at 250 ºC for 30 min prior to use. Sample vials were preheated in the agitator for 5 min before analysis, and the SPME fiber was then exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 5 min at 30 ºC under agitation, the fiber was withdrawn into the needle and immediately desorbed at 200 ºC for 2 min into the GC injection port. After each run, the fiber was baked at 250 ºC for another 3 min to eliminate some high boiling point impurities present in biological samples. GC-MS analyses were carried out on an Agilent 6890 gas chromatograph coupled with a model 5973 mass selective detector (Palo Alto, CA, USA). The GC was equipped with a 0.75 mm i.d. SPME liner. Separation of the analytes was obtained on a ZB-5MS column (Phenomenex, Torrance, CA, USA: 30m×0.25mm i.d., 0.25μm film thickness) using helium as a carrier gas (flow rate, 1 ml/min). The GC injection port and interface transfer line were maintained at 200 and 280 ºC, respectively. During the fiber desorption process, the splitless mode of injection was operated. After 2.5 min, the split vent valve opened to sweep any residual vapors from the liner. The oven temperature was initially held at 35 ºC for 3 min, then increased to 100 ºC at 10 ºC /min, and held for 2 min. For higher doses (2.5 and 5.0 mg/kg), the mass spectrometer was operated in positive electron ionization (EI) mode with an electron energy of 70 eV. Quantitation of TCE was performed using selected-ion monitoring (SIM) mode by monitoring m/z 130 (quantitation ion), m/z 132 and m/z 134 (confirmation ions). For lower doses (1, 0.1, 0.01, 0.001 and 0.0001 mg/kg), the mass spectrometer was operated in negative chemical ionization (NCI) mode with an electron energy of 235 eV. Ultra high purity (UHP) methane was used as the reagent gas at a flow rate of 2 ml/min. The ion source temperature was set at 150 ºC.

Quantitation of TCE was performed using selected-ion monitoring (SIM) of m/z 35 and 37. A solvent delay of 1.5 min was set to protect the filament from oxidation.

Calibration curves (0.25-100 ng/ml for the EI method or 0.025-25 ng/ml for the NCI method) were prepared each day when samples were analyzed. The lower limit of quantitation for TCE in blood for the EI method was 0.25 ng/ml. The NCI method showed higher sensitivity with the lower limit of quantitation at 0.025 ng/ml. Both the EI and NCI method showed good linearity with correlation coefficient (R^2) values higher than 0.99. The precision and accuracy for intra-day and inter-day measurements for both EI and NCI methods were less than 10%. The relative recoveries of TCE respect to deionized water were greater than 55%. Stability tests indicated that blood samples in the autosampler at room temperature showed no significant loss during 8 h with the relative error lower than 10% for both the EI and NCI methods. Blood samples were stored in the refrigerator at 4 ºC and analyzed within 8 hours.

Toxicokinetics data analysis. Means and standard error of the mean (SE) were calculated with Microsoft Excel 2003 (Microsoft Co., Redmond, WA). TK parameters, including area under the blood TCE concentration versus time curve (AUC_0^{∞}), volume of distribution (Vd), clearance (CL), and terminal elimination half-life ($t_{1/2}$), were calculated using WinoNonlin (ver. 4.1) noncompartmental model analysis by Scientific Consulting, Inc. (Cary, NC, USA). The maximum blood concentration (Cmax) and time of maximum blood concentration after dosing (Tmax) were observed values. Bioavailability (F) was calculated using the equation: $F= (AUC_{PO}/AUC_{IV.})(Dose_{IV}/Dose_{PO})$. Differences among the pharmacokinetic parameters of different dosage groups were evaluated by One-Way ANOVA. A value of $p < 0.05$ was considered statistically significant.

RESULTS

TCE Toxicokinetic Analyses and Characterization of Linear Kinetics

TCE blood concentration versus time profiles for rats given 3 different IV doses were obtained (Figure 7.1). TCE was quickly distributed to body tissues, resulting in a rapid decrease in TCE concentrations in the blood. For all three IV doses the rats exhibit similar elimination profiles. Toxicokinetic parameters obtained following IV injection of TCE are presented in Table 7.1. The systemic elimination for 0.1 mg/kg was slower than the other two doses, as reflected by the $t_{1/2}$, which was longer than the $t_{1/2}$ observed at the 1 and 2.5 mg/kg doses. But there was no significant difference in the half life at dose of 1 and 2.5 mg/kg. At the low dose, 0.1 mg/kg, the blood samples could easily be influenced by environmental TCE. The environmental TCE present in food, drinking water or air resulted in higher concentrations of TCE during the elimination phase and prolonged the process to eliminate TCE from the body. This effect was more important for low doses because of the extremely low concentrations of TCE in the body following low-level exposures. In order to eliminate the risk of environmental exposure to TCE, boiled tap water was provided to the animals. However, TCE at ppm levels was present in the rat food which was provided to rats 1 hour post dosing potentially resulted in elevated concentrations of TCE. Clearance (Cl_s) ranged from 49.7 to 68.8 ml/min/kg and was not significantly different in the dosage range of 0.1 to 2.5 mg/kg. The AUC increased proportionately with dose. These findings indicate that TCE exhibits linear kinetics in the dosage range from 0.1 to 2.5 mg/kg.

TCE blood concentration versus time profiles in rats administered different oral doses were pictured in Figure 7.2. It was obvious that TCE was absorbed quickly, as the T_{max} was observed at the first time point at 5 min. Similar to IV doses, TCE was quickly distributed to

body tissues, resulting in a rapid decrease in TCE concentrations in the blood. The 10, 2.5, 1 and 0.1 mg/kg rats exhibited similar elimination profiles. TCE concentrations were measurable at the 0.001 mg/kg dose for only the first 15 min post dosing. At 0.01 mg/kg, TCE was detectable for 120 min. No terminal elimination phase was observed due to concentrations in the elimination phase being below the LOQ. The complete elimination phase could not be obtained for these two doses (0.01 and 0.001 mg/kg) animals, due to the extremely low levels of TCE in their blood. Nevertheless, the profiles for these two low doses had absorption and distribution phases that were similar to those for the higher doses. Toxicokinetic parameters obtained for oral administration of TCE are presented in Table 7.2. When compared to the higher doses (e.g., 1, 2.5 and 10 mg/kg), the half-life for 0.1 mg/kg dose was significantly higher but there was no significant difference in the PK parameters between oral and IV administration at the same dose. The possible explanation for this increased half-life at low TCE doses following oral administration was similar to IV dosing which was discussed previously. In the dose range from 0.1 to 10 mg/kg, no significant difference was observed in T_{max}, clearance and volume distribution. The C_{max} and AUC increased proportionally with the dose in the dose range from 0.1 to 2.5 mg/kg. However, for the 10 mg/kg oral dose, the C_{max} increased about 8- fold and AUC increased about 6- fold when compared to the 2.5 mg/kg dose. Since the clearance of TCE was constant over the dosage range of 0.1 to 10 mg/kg, the disproportionate increase in C_{max} and AUC is due primarily to saturation of first-pass metabolism which led to a significant increase in bioavailability for the 10 mg/kg dose.

Presystemic Elimination of TCE

Bioavailability (F) was calculated using the equation: $F = (AUC_{PO}/AUC_{IV.})(Dose_{IV}/Dose_{PO})$ and was presented in Figure 7.3. Bioavailability ranged from 12.5 to 25.5 %. It

was constant in the dose range from 0.1 to 2.5 mg/kg but increased about 2- fold at the 10 mg/kg oral doses over the lower doses. The total first pass effect did not show significant differences in the low doses (0.1 to 2.5 mg/kg) but it appeared saturated as the dose increased to 10 mg/kg.

DISCUSSION

Relatively little is known about the toxicokinetics of TCE in rats following environmentally-relevant exposures. The time course data and TK parameter estimates presented here provide an overview of elimination of IV and oral administered TCE in adult rats. The key contribution of this work was the linear kinetics of TCE following extremely low levels of TCE exposures.

TCE was previously reported to be rapidly and completely absorbed from the GI tract of fasted rats, with the C_{max} occurring 6-10 min postdosing (D' Souza *et al.*, 1985). Up to 98% of administrated radiolabel was recovered from the expired air and urine of mice and rats gavaged with [^{14}C] TCE (Prout *et al.*, 1985; Dekant *et al.*, 1986). Therefore, elimination/metabolism of TCE in the GI tract can be considered to be negligible. TCE's lipophilicity, lack of charge, and low molecular weight facilitates its rapid passive diffusion across membranes of the GI tract, blood vessels, and other tissues. Following exposures, TCE is rapidly distributed into tissues resulting in a quick decrease in blood concentrations. As described in the Results section, linear kinetics were observed at the low doses studied here. Clearance was found to be constant across the dosage range from 0.1 to 2.5 mg/kg. The C_{max} and AUC changed proportionally with dose. In a previous study of high level exposures of TCE, nonlinearity was observed in TCE kinetics. Lee *et al.* (1996) reported that $t_{1/2}$ progressively increases as the dose of TCE increased. There was a disproportionate increase in AUC over the dosage range of 0.17 to 16 mg/kg. Toxicokinetic

parameters for oral doses lower than 2 mg/kg were not included in their studies. As reported in Lee's paper, doses higher than 8 mg/kg saturated the metabolic enzymes in the liver, which resulted in nonlinear elimination of TCE. At lower oral doses (e.g., 0.17 to 2 mg/kg), the toxicokinetic behavior of TCE was not included, due to the lack of sensitivity of their analytical method (LOQ: 20 ng/ml). The LOQ for the analytical method used in the work presented here was 0.025 ng/ml. This high sensitivity of the method made it possible to directly assess the kinetics and efficiency of presystemic elimination of trace levels of TCE.

In oral administration studies, bioavailability ranged from 12.5 to 25.5 % in the dose range of 0.1 to 10 mg/kg. It was constant in the dose range from 0.1 to 2.5 mg/kg but increased about 2- fold at the 10 mg/kg oral doses over the lower doses. The capacity-limited route of first-pass elimination can be expressed by Michaelis-Menten kinetics. The Michaelis-Menten, or half-saturation constant (K_m) was estimated to be 2.68 µg/mL (Lee *et al.*, 1996). K_m is an important parameter that denotes what doses and blood levels are required to produce metabolic saturation. In the oral dose range of 0.1 to 2.5 mg/kg, TCE blood concentrations did not exceed the K_m. For three IV doses, only C_0 at 2.5 mg/kg dose was slightly higher than K_m (3.81 versus 2.68 µg/mL). It demonstrated that at low doses, metabolic enzyme activity was not saturated and the liver was capable of removing most of the TCE from the body. At high dose, 10 mg/kg, the bioavailability increased about 2- fold as compared with lower doses which means the total first-pass effect was decreased significantly. The saturation of the metabolic enzymes during the first-pass may occur between dose 2.5 and 10 mg/kg. As reported by Lee *et al.* (1996), 8 mg/kg oral dose was the saturate dose for the enzymes. At the low doses (<2.5 mg/kg), the liver and lungs played an important role in eliminating TCE that has been absorbed from the GI tract. The total first-pass effect was from 83.6 to 87.5%. Before entering arterial circulation and being distributed to

tissues throughout the body, a significant proportion of low oral doses of TCE were removed by hepatic metabolism and exhalation. At doses higher than saturation dose, hepatic metabolism was saturated and nonlinear first-pass effect was observed. There have been relatively little definitive information on the capacity, or efficiency of presystemic elimination of ingested VOCs. It has been theorized but not demonstrated experimentally, that low oral doses of VOCs are completely removed by presystemic elimination. Andersen (1981), on the basis of studies of the systemic uptake of inhaled halocarbons by rats, concluded that the liver is capable of removing virtually all of a halocarbon presented to it, when the dose is not high enough to saturate metabolism. Here, we have experimentally demonstrated that a high percentage of low oral doses can be removed by the liver and lungs, but that some TCE reaches arterial circulation.

Levels of TCE found in food and drinking water are usually in the ppb range, rarely exceeding a few ppm. According to a survey by the National Water-Quality Assessment (NAWQA) program, 5% of wells throughout the United States have detectable levels of TCE, with concentrations ranging from 0.02 ug/L to 230 ug/L (Moran *et al.*, 2007). At extremely low doses studied in this paper, TCE concentrations were measurable at the 0.001 mg/kg dose for only the first 15 min post dosing and were not detectable at the 0.0001 mg/kg dose. A miniscule amount of TCE (AUC_0^{15} = 0.9 ng·min/ml) reached the systemic circulation of rats ingesting 0.001 mg/kg. This external dosage would be ingested by a 70 kg person who consumed 2 L of water containing 35 ppb TCE. Although the relative capacity of rats and humans to metabolize TCE has not been clearly established, TCE is known to be extensively metabolized by rats and by humans. Daily doses of TCE ingested in the environmental media by humans are far below levels required to saturate TCE metabolism. Therefore, most TCE consumed in food and water is

removed from the venous blood by first-pass elimination, and does not reach extrahepatic organs.

In conclusion, TK parameters could be ascertained for the 0.1-10 mg/kg doses. Bioavailability ranged from 12.5 to 25.5 %, and was constant in the dose range from 0.1 to 2.5 mg/kg but increased about 2- fold at the 10 mg/kg oral doses over the lower doses. Clearance ranged from 49.7 to 70.1 ml/min·kg and was not significantly different over this dosage range. Linear kinetics but nonlinear first-pass elimination of TCE was observed. TCE concentrations were measurable at the 0.001 mg/kg dose for only the first 15 min post dosing and were not detectable at the 0.0001 mg/kg dose. At 0.01 mg/kg, TCE was detectable for 120 min. No terminal elimination phase was present. A miniscule amount of TCE (AUC_0^{15} = 0.9 ng·min/ml) reached the systemic circulation of rats ingesting 0.001 mg/kg. This external dosage would be ingested by a 70 kg person who consumed 2 L of water containing 35 ppb TCE. The first-pass elimination played an important role in reducing arterial blood concentrations of TCE, and presumably in reducing extrahepatic tissue levels and potentially adverse effects from environmentally-relevant exposures. The results obtained in this work may have important implications in risk assessment of trace levels of TCE in the environment.

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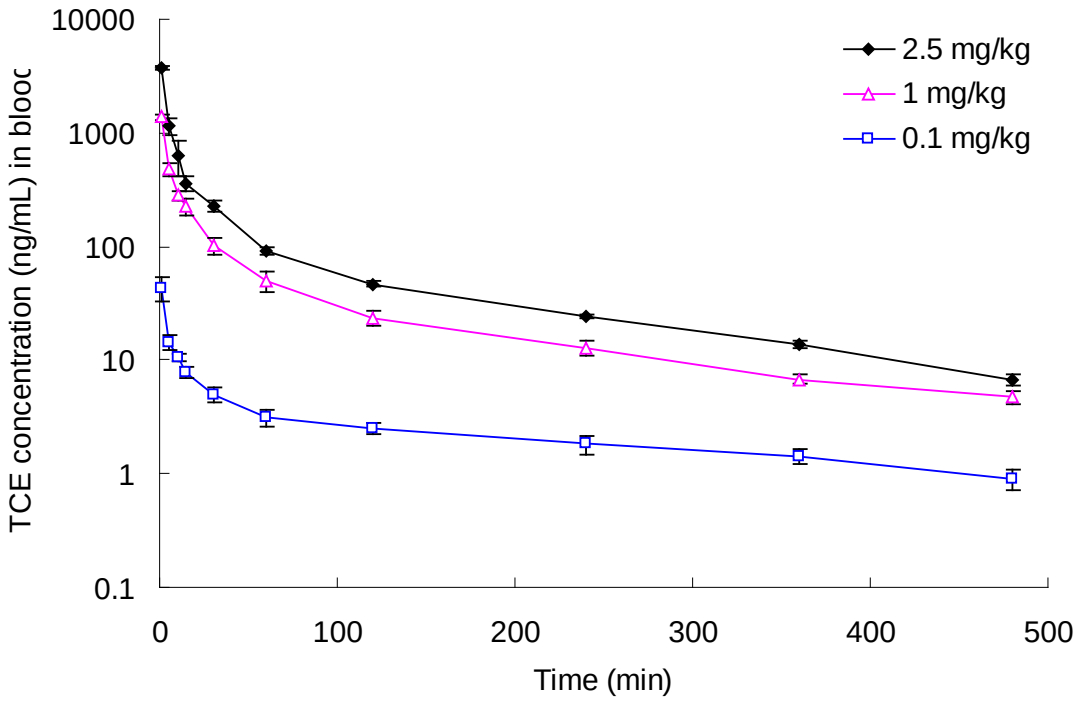

Figure 7.1. TCE blood concentration versus time profiles of S-D rats dosed intravenously with 0.1, 1 and 2.5 mg/kg of TCE (mean concentration ± standard error of the mean (SEM), n=5-7 for each time point)

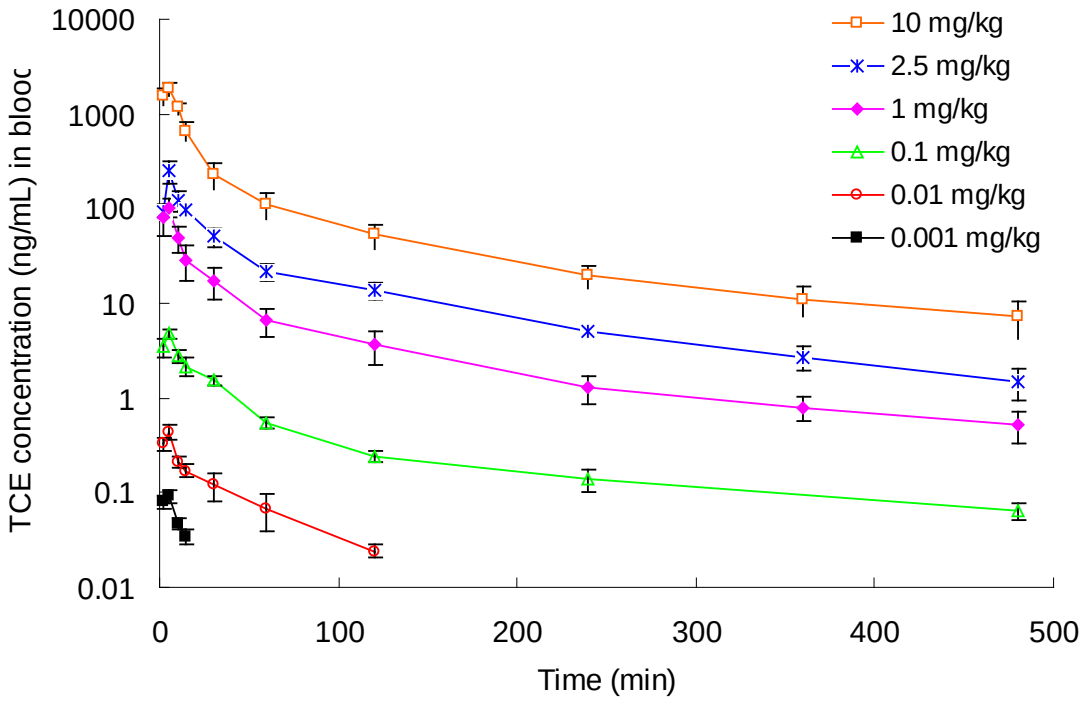

Figure 7.2. TCE blood concentration versus time profiles of S-D rats dosed orally with 0.001, 0.01, 0.1, 1, 2.5 and 5 mg/kg of TCE (mean concentration ± standard error of the mean (SEM), n=5-6 for each time point)

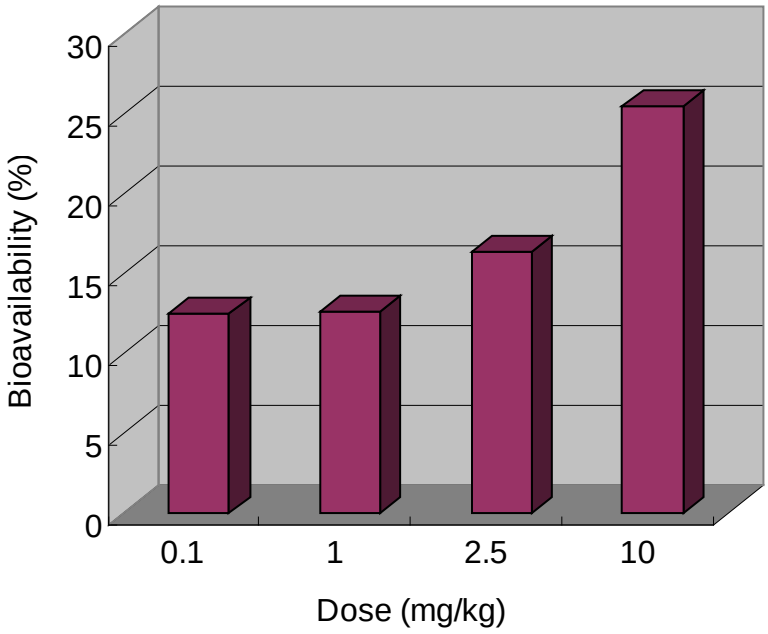

Figure 7.3. Bioavailability (%) data for TCE in the dosage range 0.01-5.0 mg/kg.

Table 7.1. Toxicokinetc parameters† of TCE in SD rats (IV administration)

Toxicokinetic parameters	0.1 mg/kg	1 mg/kg	2.5 mg/kg
Half life (min)	201.4 ± 63.0[a]	157.5 ± 28.3	135.5 ± 18.9
Clearance (mL/min/kg)	68.8 ±24.1	49.7 ± 8.5	58.4 ± 9.2
Vd (mL/kg)	18.6 ± 4.2[a]	13.5 ± 5.8	11.0 ± 2.4
AUC (µg•min/mL)	1.6 ± 0.5	20.6 ± 3.7	43.7 ± 6.8

† Values represent means ± standard deviation for groups of 5-7 rats.
[a] indicates significant difference

Table 7.2. Toxicokinetc parameters† of TCE in SD rats (oral administration)

Toxicokinetic parameters	0.1 mg/kg	1 mg/kg	2.5 mg/kg	10 mg/kg
C_{max} (ng/mL)	4.9 ± 1.2	107.8 ± 59.9	250.4 ± 144.3	1916 ± 610
T_{max} (min)	4.4 ± 1.3	4.4 ± 1.3	6 ± 2.2	3.8 ± 1.6
Half life (min)	209.4 ± 56.9[a]	134.6 ± 20.5	106.7 ± 30.4	125.7 ± 38.9
Clearance (mL/min/kg)	64.0 ± 10.7	69.8 ± 40.8	67.8 ± 31.5	70.1 ± 35.7
Vd (mL/kg)	19.5 ± 7.4	13.7 ± 8.1	9.6 ± 2.5	12.5 ± 7.9
AUC (µg•min/mL)	0.20 ± 0.03	2.6 ± 1.6	7.1 ± 2.9	44.4 ± 21.3

† Values represent means ± standard deviation for groups of 5-7 rats.
[a] indicates significant difference

CHAPTER 8

CONCLUSIONS

Trichloroethylene (TCE) is a significant groundwater contaminant and is presently an important issue in human health risk assessment. While TCE produces a number of toxic responses, including tumors, at high doses in laboratory animals, its ability to induce cancer in humans is debatable. Laboratory studies have indicated that the carcinogenicity of TCE in rodents is due to the actions of toxic metabolites rather than the parent compound. TCE metabolites, trichloroacetic acid (TCA) and dichloroacetic acid (DCA), have themselves been shown to produce liver tumors in mice and/or rats. Concentrations of TCE in drinking water supplies are usually very low. Nevertheless, there is concern on the part of the EPA that even trace levels may present a cancer risk to humans. The human body possesses a number of defense mechanisms to protect against low-level toxic and mutagenic insults. First-pass, or presystemic elimination is one such mechanism. Drugs and chemicals absorbed from the gastrointestinal tract must first pass though the portal blood into the liver and on to the lungs, before entering arterial circulation and being transported to tissues throughout the body. It has been theorized but not demonstrated experimentally, that low oral doses of VOCs are completely removed by presystemic elimination. It is important to directly measure the capacity of first-pass elimination of TCE under environmental exposure conditions. The bioanalytical methods presented here are simple, sensitive and specific for the determination of TCE and its metabolites in multiple blood and tissue samples**.**

SPME GC-EIMS methods were developed for the determination of TCE in rat plasma, blood, liver, lungs, kidney, brain and fat. SPME is a method of sample extraction that is growing in popularity. More scientists are beginning to apply this technique because of its simplicity and ease of use. SPME combines sampling, extraction, concentration and sample injection in one step. SPME conditions were optimized for each matrix to enhance the extraction efficiency. For electrophilic atoms (halogens), negative chemical ionization (NCI) mass spectrometry has increased selectivity and sensitivity. A SPME GC-NCIMS method was therefore optimized and validated for the determination of TCE in rat blood and tissue samples. Under the same SPME conditions, the NCI method significantly increased the sensitivity with an LOQ 10 times lower than the EI method.

Methods are also presented for the analysis of TCE metabolites, i.e., trichloroethanol (TCOH), DCA and TCA in rat blood. The analytical procedure involves derivatization of TCA and DCA to their ethyl esters with acidic ethanol, followed by headspace sampling using SPME GC-NCI-MS determination. Parameters affecting both derivatization efficiency and the headspace SPME procedure were optimized to achieve maximum sensitivity. In comparison with other derivatization reagents, acidic ethanol was simpler, safer and more efficient. Most importantly, this validated method did not involve the decarboxylation of TCA and the subsequent conversion of TCA to DCA, which usually occurred with other GC derivatization methods for TCA and DCA.

Using these sensitive, selective, and validated analytical methods developed above, the toxicokinetic (TK) behavior of TCE following ingestion of environmentally-relevant (i.e., trace) doses was characterized. TCE exhibited linear kinetics in rats following low levels of oral administrations. The capacity of the presystemic elimination of trace levels of TCE in rats was

estimated. These data are essential for the development and validation of a PBPK model which would be used to predict the ability of humans to eliminate TCE. This would improve the accuracy of the assessment of human health risk due to TCE exposure.