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In matrix derivatization of trichloroethylene metabolites in human plasma with methyl chloroformate and their determination by solid-phase microextraction–gas chromatography-electron capture detector

Mohana Krishna Reddy Mudiam[a,*], Rajeev Jain[a,b,1], Meenu Varshney[c,1], Ratnasekhar Ch[a], Abhishek Chauhan[a], Sudhir Kumar Goyal[c], Haider A. Khan[b], R.C. Murthy[a]

[a] Analytical Chemistry Section, CSIR-Indian Institute of Toxicology Research, M G Marg, Lucknow 226001, UP, India
[b] Department of Medical Elementology and Toxicology, Jamia Hamdard, Hamdard Nagar, New Delhi 110006, India
[c] Petroleum Toxicology, CSIR-Indian Institute of Toxicology Research, M G Marg, Lucknow 226001, UP, India

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ABSTRACT

Trichloroethylene (TCE) is a common industrial chemical that has been widely used as metal degreaser and for many industrial purposes. In humans, TCE is metabolized into dichloroacetic acid (DCA), trichloroacetic acid (TCA) and trichloroethanol (TCOH). A simple and rapid method has been developed for the quantitative determination of TCE metabolites. The procedure involves the *in situ* derivatization of TCE metabolites with methyl chloroformate (MCF) directly in diluted plasma samples followed by extraction and analysis with solid-phase microextraction (SPME) coupled to gas chromatography-electron capture detector (GC-ECD). Factors which can influence the efficiency of derivatization such as amount of MCF and pyridine (PYR), ratio of water/methanol were optimized. The factors which can affect the extraction efficiencies of SPME were screened using 2^{7-4} Placket–Burman Design (PBD). A central composite design (CCD) was then applied to further optimize the most significant factors for optimum SPME extraction. The optimum factors for the SPME extraction were found to be 562.5 mg of NaCl, pH at 1 and an extraction time of 22 min. Recoveries and detection limits of all three analytes in plasma were found to be in the range of 92.69–97.55% and 0.036–0.068 $\mu g\,mL^{-1}$ of plasma, respectively. The correlation coefficients were found to be in the range of 0.990–0.995. The intra- and inter-day precisions for TCE metabolites were found to be in the range of 2.37–4.81% and 5.13–7.61%, respectively. The major advantage of this method is that MCF derivatization allows conversion of TCE metabolites into their methyl esters in very short time ($\leq$30 s) at room temperature directly in the plasma samples, thus makes it a solventless analysis. The method developed was successfully applied to the plasma samples of humans exposed to TCE.

1. Introduction

Trichloroethylene (TCE) is a volatile organic chemical mainly used as industrial solvent in automotive and metal industries for vapor degreasing and cold cleaning of metal parts. The wide use of TCE by the workers of metal industries for cleaning purposes leads to high exposure of TCE. Dichloroacetic acid (DCA), trichloroacetic acid (TCA) and trichloroethanol (TCOH) are three major metabolites of TCE. DCA and TCA are additionally encountered in drinking water as chlorination disinfection by-products [1]. Animal studies demonstrated the carcinogenicity of TCE through its metabolite activation in mice liver [2,3].

Quantitative determination of TCE and its major metabolites in biological matrices will be used as a biomarker of TCE exposure which is necessary for medical and clinical monitoring and to evaluate their carcinogenicity. Several analytical methods have been reported for the determination of TCE metabolites. The first method was based on Fujiwara reaction and spectrophotometric determination [4]. Ion-exclusion chromatographic method was reported for the direct determination of TCOH in plasma and urine samples, however the LOQ of the method was

* Corresponding author. Tel.: +91 9454985887; fax: +91 5222611547.
E-mail address: mohanitrc@gmail.com (M.K.R. Mudiam).
[1] Both authors are equally contributed.

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very high (3 mg L^{-1}) [5]. Gas chromatographic determination of TCE metabolites requires derivatization due to their low volatility and polar nature. Derivatizing reagents such as BF_3–methanol, diazomethane, acidic methanol and ethanol [6–9] have been often used for the derivatization of TCE metabolites, but these reagents require longer reaction time and elevated reaction temperatures.

Alkyl chloroformates (ACF) are known for years as rapid esterification reagents in aqueous medium [10]. The remarkable advantages of ACF derivatizing reagent over the other derivatization techniques are, (a) the reaction can perform directly in aqueous medium, (b) derivatization is completed in less than a minute, (c) can occur at room temperature and (d) the non-polar derivatives formed can directly extracted with SPME [11]. ACF derivatization has been widely employed for the conversion of polar compounds to their non-polar derivatives. Analytes containing different polar functional groups such as phenolic hydroxy, carboxyl, amines and amino acids are derivatized with ACFs [12–14].

Determination of TCE metabolites in biological samples like plasma samples requires extensive sample preparation before analysis. Liquid–liquid extraction has been widely used for the extraction of derivatives of TCE metabolites [8]. In recent years, SPME has also been applied for the same purpose due to its advantages like solvent free system and requires very few amount of sample [9,15,16]. The extraction factors of SPME such as extraction temperature and time, desorption temperature and time, pH, ionic strength and head space volume were screened by multivariate strategy based on an experimental design using a Placket–Burman Design (PBD). The SPME factors screened were further optimized using central composite design (CCD) approach. So far, no analytical procedure based on MCF derivatization has been reported for the determination of TCE metabolites in human plasma samples. In the present study, we report a simple and rapid analytical method for the determination of TCE metabolites in human plasma samples based on *in matrix* derivatization with MCF followed by SPME extraction and GC-ECD analysis.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade unless otherwise stated. The standard DCA, TCA and TCOH were purchased from Sigma (St. Louis, MO, USA). MCF, pyridine and all other solvents used in this study were procured from Merck (Darmstadt, Germany). The SPME holder and polydimethylsiloxane (PDMS) fiber (100 μm film thickness), extraction vials, septa and aluminum caps were obtained from Supelco (Bellefonte, USA). The SPME–PDMS fiber was conditioned at 250 °C for 30 min according to the procedure recommended by the manufacturer. Plasma samples were collected from healthy volunteers for the purpose of method validation and application. Ultra pure water produced from Milli-Q water purification system (Millipore, Bedfore, MA, USA).

Stock solution of DCA, TCA and TCOH were prepared in ultra pure water at a concentration of 1 mg mL^{-1} and stored at 4 °C until analysis. Working standard solution was prepared every time before analysis by appropriate dilution of stock solution.

2.2. Subjects and blood collection

Blood was taken through venipuncture from trichloroethylene exposed subjects from a lock industry where trichloroethylene was used as a metal degreaser. The institutional ethical committee approval was obtained to conduct the study. A detailed informed consent was obtained from each subject. The exposure of subjects was for the duration of 4–20 years. Approximately 1 mL of blood was collected from each subject in EDTA coated vials (B.D. Vacutainer). These samples were placed on ice and plasma was separated through centrifugation at the speed of 2000 × g for 5 min. Plasma was aspirated to new collection tube and stored at −80 °C until use for processing.

2.3. Chromatographic conditions

The analysis of TCE metabolites were performed on Perkin Elmer Clarus 500 gas chromatograph equipped with DB-5 (5% phenyl methyl polysiloxane, 25 m × 0.25 mm I.D. × 0.25 μm film thickness) capillary column and an electron capture detector (ECD) operated at 375 °C. High purity nitrogen (99.999%) was used as carrier gas at a flow rate of 1 mL min^{-1} and also as a makeup gas for ECD at a flow rate of 30 mL min^{-1}. The oven temperature was programmed initially from 80 °C to 100 °C at a rate of 2 °C min^{-1} (hold for 3 min), it was further increased up to 280 °C at a rate of 45 °C min^{-1} and was held for 5 min (total run time 22 min). The GC injector port was held at 200 °C to allow complete vaporization of analytes and operated in split mode at split ration of 1:5 [9].

The confirmation of derivatization of TCE metabolites with MCF was achieved by analyzing the derivatives of TCE metabolites standard dissolved in Milli-Q water on Trace GC Ultra coupled to TSQ Quantum XLS mass spectrometer (Thermo Scientific, FL, USA) equipped with Elite-5MS capillary column (60 m × 0.25 mm i.d × 0.25 μm film thickness of 5% phenyl and 95% methylpolysiloxane). The GC oven was kept at an initial temperature of 50 °C for 5 min and increased at a rate of 10 °C min^{-1} up to 100 °C and held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. One μL of the derivatized sample was injected at an injector temperature of 200 °C. The mass spectrometer was operated using electron impact (EI) ionization mode (70 eV). Transfer line and source temperature were kept at 290 and 220 °C, respectively. The derivatization products were confirmed by comparing the mass spectra obtained from standard to their mass spectras available in instrument library i.e. NIST library.

2.4. Statistical data handling and processing

Designs of experiments (PBD and CCD) were constructed and the results were evaluated using the statsoft statistical software package "Statistica 10.0" (Tulsa, OK, USA). Calculations of optimized responses were based on the sum of the area of all the peaks obtained during GC–MS analysis [17,18].

2.5. Derivatization and extraction

The derivatization of TCE metabolites were carried directly in plasma samples after diluting with ultrapure water. An aliquot of plasma sample (100 μL) was mixed with 500 μL of methanol and 500 μL of water. To the resultant mixture, 200 μL of PYR was added as a catalyst followed by the addition of 150 μL of MCF twice (a total of 300 μL) in order to ensure complete derivatization of TCE metabolites and the reaction carried at room temperature for 30 s. The derivatized sample was then placed in 10 mL SPME vial and diluted with ultrapure water up to 3 mL to get the head space volume of 7 mL. The pH of the sample at this stage was 1 and adjusted with 5 M NaOH for optimization studies using design of experiments. To enhance the ionic strength of the sample 562.5 mg of NaCl was added. Then the sample was exposed to PDMS fiber for 22 min at 50 °C. After extraction of non-polar derivatives of TCE metabolites, the fiber was pulled back into the needle and inserted into the GC injector port for desorption of analytes for 1 min at 200 °C.

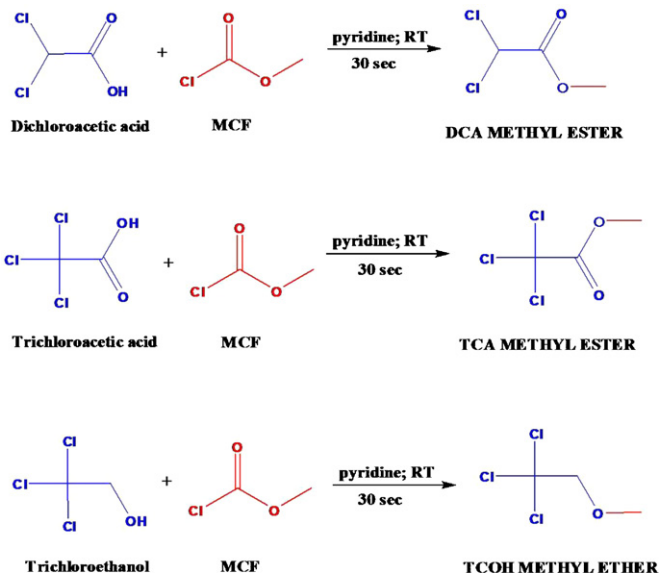

Fig. 1. Reaction scheme of TCE metabolites with MCF.

2.6. Validation study

A linear regression calibration of TCE metabolites were performed based on peak areas directly in plasma samples at five different concentrations. Control plasma samples were spiked at 0.15, 0.25, 0.5, 0.75 and 1 μg mL^{-1} for DCA and TCOH; whereas for TCA, plasma samples were spiked at 0.25, 0.35, 0.5, 1 and 1.5 μg mL^{-1}, respectively. The intra and inter-day precision were expressed as percent relative standard deviation (%RSD) and were studied at three different concentration levels (i.e. 0.15, 0.5 and 1 μg mL^{-1} for DCA and TCOH whereas 0.25, 0.5 and 1.5 μg mL^{-1} for TCA) of calibration graphs for each analyte with five replicates. The sensitivity of the method was described as limit of detection (LOD) and limit of quantification (LOQ). The relative recoveries were calculated for TCE metabolites by comparing the amount extracted from spiked plasma samples with the amount extracted from spiked water samples at same concentrations.

3. Results and discussion

The derivatization of TCE metabolites with MCF has several advantages over other derivatization techniques reported earlier. MCF derivatization can take place directly in aqueous medium in the presence of PYR within a minute at room temperature, which saves the sample preparation time and running cost of the analysis. Further, the non-polar analytes can easily be extracted with solvent less SPME technique. Factors which greatly affect the derivatization efficiency of TCE metabolites such as amount of MCF, PYR and volume ratio of water/methanol were optimized by one variable at a time (OVAT) approach. For the best extraction efficiency of TCE metabolites with SPME, screening and optimization of the SPME factors is required. It is fulfilled by two step surface design approach, PBD for screening of most significant factors and CCD to optimize these factors.

3.1. Optimization of derivatization conditions

The derivatization reaction scheme of TCE metabolites with MCF is depicted in Fig. 1. Derivatization was carried out in 1 mL of reaction medium consisting of water and methanol. Non polar derivatives thus formed were extracted with 1 mL of *n*-hexane and 1 μL was injected into GC-ECD for analysis. Initially, the volume ratio of reaction mixture i.e. water and methanol was optimized in order to get good derivatization yield. For this purpose, a set of experiments were conducted at three different water/methanol ratio of 0:1, 1:1 and 1:0 *v*/*v* (with a total volume 1 mL). Good detector responses were obtained when the ratio of water and methanol is equal to 1:1 (*v*/*v*; 500 μL each of water and methanol). At water/methanol ratio of 1:0 (*v*/*v*), a drastic and unusual increase in peak intensity of TCA was observed with significant decrease in the peak intensities of DCA and TCA. It is also notable that at this ratio (i.e. 1:0, *v*/*v*) of water/methanol, the precision for TCE metabolites was found to be poor. Therefore, not to sacrifice the overall sensitivity and precision of the method, a ratio of 1:1 (*v*/*v*) was selected as reaction medium. Another experiment was performed to optimize the volume of MCF in which five different volumes in the range of 100–500 μL at an interval of 100 μL were checked for better derivatization yield. Detector response for DCA was increased up to 300 μL and then reached a plateau, whereas detector response for TCA was increased as the volume of MCF increased; TCOH does not show any specific trend. Using MCF more than 300 μL, the chromatograms obtained were too noisy and the precision were also poor which may be due to the formation of other products as the sample (i.e. plasma) is too complex in nature. Therefore, 300 μL of MCF was selected for further experiments. Volume of PYR was also screened in the range from 100–500 μL at an interval of 100 μL. PYR at a volume of 200 μL was found to be optimum to catalyze the reaction.

Table 1
Factors, codes, low level and high levels in Placket–Burman design matrix.

Factor	Levels	
	Low (−1)	High (+1)
(Des T °C) Desorption temperature (°C)	150	250
(Ext T °C) Extraction temperature (°C)	40	80
(Extt) Extraction time (min)	5	30
Ionic strength (% *w*/*v*)	0	20
pH	2	9
(HS volume) Head space volume (mL)	3	10
(Dest) Desorption time (min)	0.5	3

3.2. Screening design

PBD is a best design for rapidly screening more number of variables from a multi-variant system. In this design the interactions can be completely ignored so that the main effects are calculated with a reduced number of experiments. At 2 levels with k factors the design consists of $k+1$ runs where k is number of factors. In this experiment seven factors are selected which gives 8 runs. To reduce the experimental error in statistical interpretation, the experiments were carried out in three replicates in a random manner. Overall the matrix consists of 24 runs and the experimental factors were screened at 2 levels i.e. low (−1) and high (+1). The seven factors and their levels are depicted in Table 1. The results obtained were evaluated by Analysis of Variance (ANOVA). According to results, pH is the most significant factor with negative effect. Ionic strength is the next most significant factor with positive effect. Extraction time and head space volume was the next significant factors. Out of these the most significant factors pH, ionic strength and extraction time were selected for further optimization using CCD. Head space volume of 7 mL was selected based on the fact that, this volume is sufficient for equilibration of the analyte between fiber and sample volume. The values of less significant SPME factors such as desorption time, desorption temperature and extraction temperature were fixed at 1 min, 200 °C and 50 °C, respectively.

Table 2
Factors and their levels for 2^3 central composite design.

Factors	Levels			Star points $\alpha = 1.68$	
	Low (−1)	Central (0)	High (+1)	$-\alpha$	$+\alpha$
pH	3	6	9	0.9	11
NaCl (mg)	120	285	450	7.5	562.4
Ext time[a] (min)	10	20	30	3.1	36.8

[a] Extraction time.

3.3. Optimization design

After screening the SPME variables with the help of PBD, next step was to optimize the values of these three factors viz. pH, ionic strength and extraction time using CCD approach combined with desirability function. CCD is one of the most commonly used designs described by Box and Wilson [19] useful to fit quadratic models. CCD involves a factorial design and a star design in which experimental points are located at a distance of $+\alpha$ and $-\alpha$ from center, and an experimental point at the center. The three factors, their levels and the matrix of such a rotatable CCD were depicted in Table 2 and Table 3. The design consists of 2^3 factorial runs with 6 axial runs and one center point with triplicate, overall the design produces 18 runs according to the following equation:

$$N = 2^k + 2^k + C_p \quad (1)$$

where, N is total number of runs, k is the factor number and C_p is the replicate number of central point, respectively [20,21]. Therefore a total of 18 experiments had to be run for the CCD (Table 3). In this step, experiments were performed in a random manner to minimize the effect of uncontrolled variables and the peak responses were expressed as area of the peak. Axial distance $\alpha = 1.68$ was selected in order to establish the rotatability condition. The data obtained in central composite design was evaluated by ANOVA. Fig. 2 shows the response surface plots obtained by the CCD design. The responses were mapped against two experimental factors while the third is held constant at its central level. The highest response was found in the region of 20–30 min for extraction time, pH below 2 and ionic strength between 400 and 600 mg of NaCl. As the ionic strength increases, the solubility of MCF derivatives of TCE metabolites in aqueous media decreases which subsequently leads to the increase in the extraction efficiency of TCE metabolites from sample on to the fiber (salting out effect). At higher pH, only TCOH is obtained and the remaining two metabolites were not extracted. As the pH reaches 6, two methyl derivatives of TCE metabolites such as DCA and TCOH were detected. As the pH remains acidic, all the three metabolites were detected. So, the maximum extraction efficiencies of all analytes were observed at lower pH values. Optimum conditions were predicted by Desirability function. Desirability function is a multi-criteria methodology mainly used to find out the satisfactory responses when optimum values are localized in different areas for all analytes. The method is based on the construction of individual desirability functions for response of each analyte and the measured responses are transformed into a dimensionless individual desirability scale which ranges from completely undesirable ($d = 0$) to fully desirable response ($d = 1$) [21]. The prediction profiles and desirability graphs are shown in Fig. 3. The pH value of 0.9, extraction time of 22 min and ionic strength of 562.5 mg of NaCl were obtained as optimum and used for further studies.

Table 3
Design matrix for 2^3 central composite design.

Runs	pH	IS[c] (% w/v)	Extt[b] (min)
1	−1	−1	−1
2	−1	−1	1
3	−1	1	−1
4	−1	1	1
5	1	−1	−1
6	1	−1	1
7	1	1	−1
8	1	1	1
9	−1.682	0	0
10	1.682	0	0
11	0	−1.682	0
12	0	1.682	0
13	0	0	−1.682
14	0	0	1.682
15 (C)[a]	0	0	0
16 (C)[a]	0	0	0
17 (C)[a]	0	0	0
18 (C)[a]	0	0	0

[a] Centre point.
[b] Extraction time.
[c] Ionic strength.

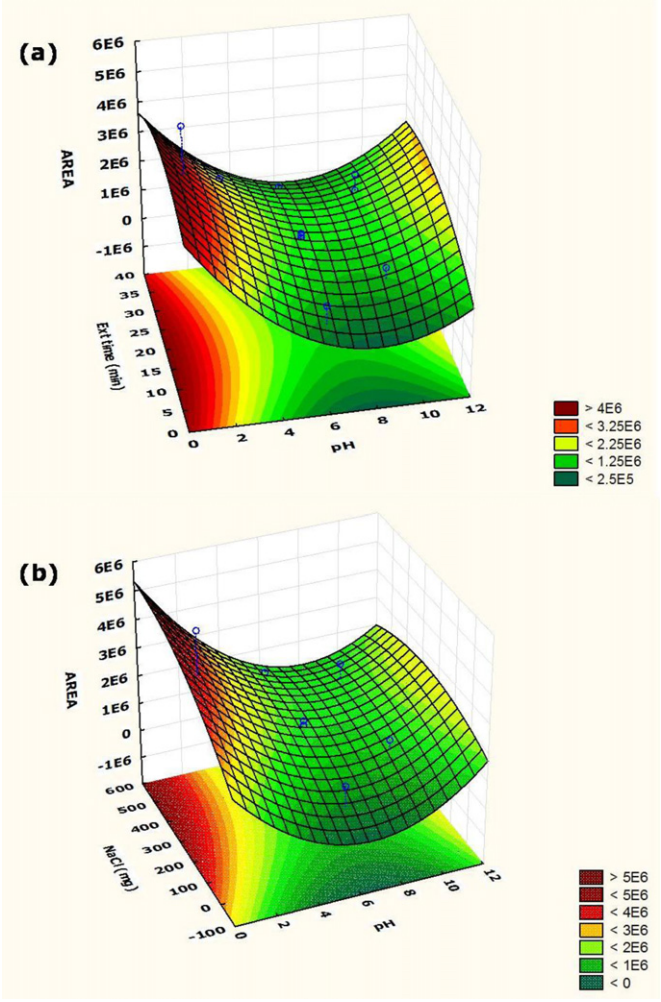

Fig. 2. Response surface for 2^3 central composite design, (a) extraction time (min) v/s pH (b) NaCl (mg) v/s pH.

Table 4
Method validation parameters and recovery assay of TCE metabolites in plasma samples.

Analyte	Linearity (μg mL^{-1})	r^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	%Recovery (%RSD)		
					0.25 μg mL^{-1}	0.5 μg mL^{-1}	1 μg mL^{-1}
DCA	0.15–1	0.990	0.036	0.118	93.27 (4.16)	92.69 (2.91)	96.45 (3.51)
TCOH	0.15–1	0.994	0.037	0.122	95.41 (3.16)	94.90 (2.14)	96.14 (2.46)
TCA	0.25–1.5	0.995	0.068	0.224	94.57 (3.48)	95.32 (3.19)	97.55 (4.11)

3.4. Analytical performance of the method

Under the optimized conditions method was evaluated for its linearity, limit of detection (LOD), limit of quantification (LOQ) and precision (intra and inter-day). The calibration graph of the proposed method in spiked plasma samples were found to be linear in the range of 0.15–1 μg mL^{-1} for DCA and TCOH, and 0.25–1.5 μg mL^{-1} for TCA, respectively (Table 4). The linear relationship between concentration and peak area was found to be acceptable with a correlation coefficient (R^2) of 0.99–0.995. The LOD and LOQ were calculated by the following expressions according to the recommendations of Eurachem guide [22,23].

$$\mathrm{LOD} = X_b + 3S$$

$$\mathrm{LOQ} = X_b + 10S$$

where, X_b is the mean peak area of blank and S is standard deviation of mean peak areas for TCE metabolites. The LOD for TCE metabolites was found to be in the range of 0.036–0.068 μg mL^{-1} whereas LOQ was found to be in the range of 0.118–0.224 μg mL^{-1} in spiked plasma samples (Table 4). Intra-day precisions were studied at 0.15, 0.5 and 1 μg mL^{-1} for DCA and TCOH, and 0.25, 0.5 and 1.5 μg mL^{-1} for TCA, respectively (n = 5) and were found to be less than 4.81% for all TCE metabolites. The inter-day precision was evaluated at the same concentration levels for five consecutive days (n = 5) and found to be less than 7.61% for all the analytes (Table 5). Relative recoveries of TCE metabolites from plasma samples were studied at three concentration levels i.e. 0.25, 0.5 and 1 μg mL^{-1}. All recovery experiments were performed in triplicate and %RSD were calculated for each recovery. Recoveries of TCE metabolites from spiked plasma samples were found to be in the range of 92.69–97.55% with an RSD of 2.14–4.16% (Table 4). Fig. 4 shows the GC-ECD chromatogram of TCE metabolites in ultra pure water and plasma samples. The formation of methyl esters of TCE metabolites after MCF derivatization was confirmed by matching the mass spectra of standards with mass spectra available in NIST library after GC–MS analysis. The mass spectra of each TCE metabolite in plasma samples were shown in Fig. 5. A comparison of the present method to other methods with respect to sensitivity has been provided in Table 6.

Table 5
Intra-day (n = 5) and inter-day precisions for TCE metabolites in plasma samples.

Concentration (μg mL^{-1})	Intra-day (%RSD)	Inter-day (%RSD)
DCA		
0.15	4.81	7.46
0.5	3.57	6.78
1	3.67	6.38
TCOH		
0.15	3.16	6.37
0.5	2.49	6.48
1	2.37	5.13
TCA		
0.25	4.16	7.61
0.5	3.67	6.59
1.5	2.55	5.98

3.5. Application to real samples

The developed method was successfully applied to the real plasma samples (11 subjects) of exposed workers working in a lock industry. Apart from the exposed samples, 10 samples were also collected as control samples from the subjects who were not exposed to TCE. Out of the exposed samples analyzed, 3 samples were found to contain all three TCE metabolites and in the remaining samples only one TCE metabolite out of three was detected (Table 7). None of the TCE metabolites were detected in control samples.

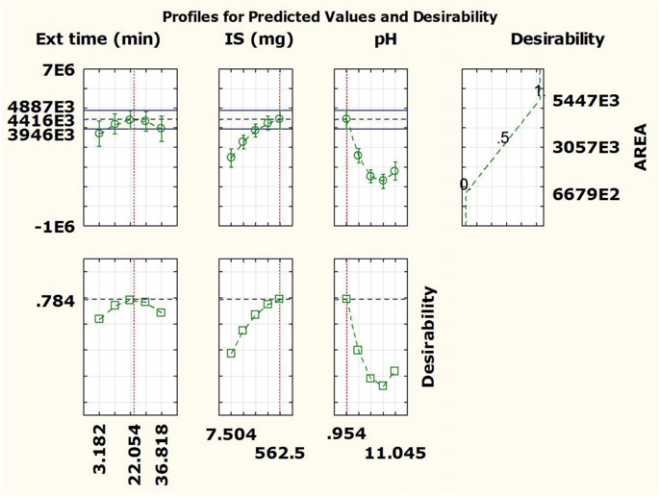

Fig. 3. Profiles for predicted values and desirability functions for peak responses of TCE metabolites. Dashed line indicated current values after optimization.

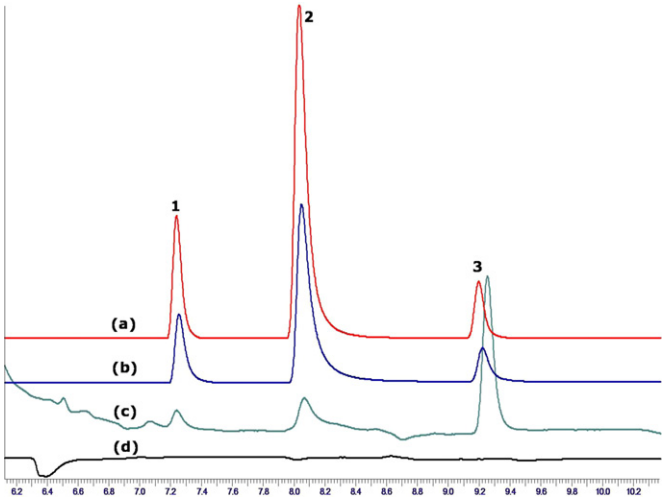

Fig. 4. GC-ECD chromatogram of TCE metabolites (a) standard, concentration 1 μg mL^{-1}, (b) plasma sample, spiked concentration 0.5 μg mL^{-1}, (c) real plasma sample of exposed worker (d) control plasma sample. Peak identification: 1, DCA methyl ester; 2, TCOH methyl ester and 3, TCA methyl ester.

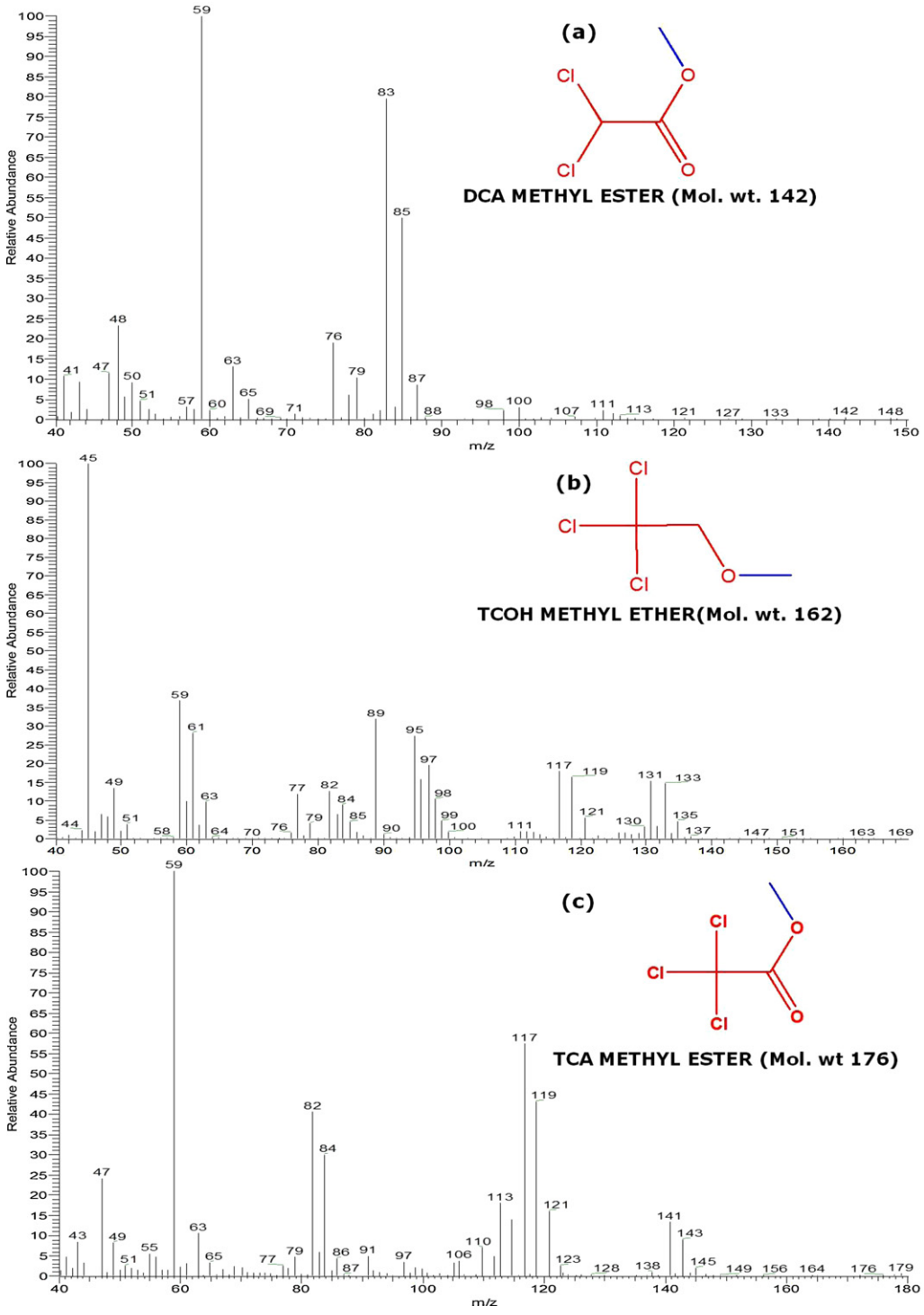

Fig. 5. Mass spectra of TCE metabolites (a) DCA methyl ester (b) TCOH methyl ether and (c) TCA methyl ester.

Table 6
Comparison of sensitivity of different methods to present method.

S. No.	LOD or LOQ ($\mu g\,mL^{-1}$)	Matrix	Analyte	Detection technique	Reference
1	3 (LOQ)	Plasma and urine	TCOH	Ion exclusion Chromatography	[5]
2	0.001 (LOQ)	Rat blood	DCA, TCOH, TCA	GC-NCI-MS	[9]
3	0.074 (LOQ)	Plasma	DCA	GC–MS	[24]
4	1.248 (LOQ)	Plasma	TCA	GC–MS	[25]
5	0.05 (LOD)	Plasma and urine	DCA	HPLC	[26]
6	0.036, 0.037, 0.068 (LOD)	Plasma	DCA, TCOH, TCA	GC-ECD	Present method

Table 7
Amount of TCE metabolites in the plasma of exposed workers after MCF derivatization followed by SPME–GC-ECD.

S. No.	DCA[b] ($\mu g\,mL^{-1}$)	TCOH[b] ($\mu g\,mL^{-1}$)	TCA[b] ($\mu g\,mL^{-1}$)
1	ND[a]	0.475	ND[a]
2	1.262	0.617	1.969
3	0.233	0.149	ND[a]
4	ND[a]	ND[a]	0.332
5	ND[a]	ND[a]	2.494
6	0.534	0.260	1.417
7	0.249	ND	ND[a]
8	ND[a]	0.311	ND[a]
9	ND[a]	ND[a]	0.517
10	0.246	ND[a]	ND[a]
11	0.163	0.144	0.333

[a] Not detected
[b] Values are based on triplicate analysis; %RSD is less than 4.3%

4. Conclusion

In the present study a rapid, simple and environment friendly method was developed for the quantitative determination of TCE metabolites viz. DCA, TCA and TCOH in plasma samples. The derivatization with MCF is found to have several advantages over other derivatization techniques like acidic methanol, ethanol and BF_3 methanol. The derivatization rapidly converts the polar TCE metabolites into their derivatives within one minute in directly plasma samples and the reaction does not require elevated temperatures. Further the non-polar derivatives can easily extracted using SPME, which is a solvent less microextraction technique and have several advantages over conventional liquid–liquid extraction. The developed method may find wide applications in various clinical and toxicological laboratories for the determination of TCE metabolites as a biomarker of TCE exposure.

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