

Green Chemistry in Urinalysis for Trichloroethanol and Trichloroacetic Acid as Markers of Exposure to Chlorinated Hydrocarbon Solvents

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Abstract: The aim of the present study was to develop a method of urinalysis for trichloroacetic acid (TCA) and trichloroethanol (TCE), and therefore total trichloro-compounds (TTC) as the sum, with least use of hazardous chemicals, being green in that sense. After acid hydrolysis followed by dilution with an ethanol (EtOH)-methanol (MeOH)-water mixture, capillary gas-chromatography with an electron-capture detector can quantify TCA and TCE in the diluted hydrolyzate. Comparison studies showed that the results were identical among three methods, i.e., 1. the method developed in the present study, 2. a head-space GC with acid hydrolysis of conjugated TCE and methyl-esterification of TCA¹, and 3. traditional colorimetry with Fujiwara reaction²). When applied to exposure - excretion analysis, the three methods gave results reproducible to each other. Over-all evaluation therefore was such that the method developed in the present study is as equally reliable as previously developed methods. It should be further noted that the procedures are very simple, with minimum use of occupationally or environmentally hazardous chemicals. In case the determination of only TCA is requested, it is possible to skip the hydrolysis step so that the treatment prior to the GC analysis is even simpler, i.e., just a 60-fold dilution of the urine sample with the EtOH-MeOH-water mixture. It was also demonstrated that correction of urinary analyte levels for urine density in terms of creatinine or specific gravity did not improve the correlation with the intensity of TRI exposure.

Key words: Green chemistry, Total trichloro-compounds, Trichloroacetic acid, Trichloroethanol, Trichloroethylene, Urinalysis

Introduction

Until very recently, trichloroethylene (TRI)^{3,4} and 1,1,1-trichloroethane (methylchloroform or MC)⁵⁻⁷ had been among the most popular chlorinated hydrocarbon solvents, and tetrachloroethylene (TETRA) is still widely used for cleaning of clothes⁸⁻¹⁰ and other materials. Trichloroethanol (TCE) and trichloroacetic acid (TCA), and therefore total trichloro-compounds (TTC) as the sum of the two, are major metabolites in urine of workers exposed to these solvents¹¹⁻¹³.

Whereas the validity of TCE and TCA as the urinary

markers of exposure to TRI, MC and TETRA has been well established not only in industrial health^{3-5, 8, 10, 11, 14, 15} but in clinical practice of solvent intoxication^{9, 16-19}, the major problem in application of TCE and TCA analyses is the use of objectionable (e.g., pyridine) or hazardous chemicals (e.g., chromium trioxide).

In the present report, an ECD-GC method for determination of urinary TCA and TCE is described, which requires no carcinogenic or objectionable chemicals but hydrochloric acid, methanol and ethanol. In this sense, the method developed requires minimum use of hazardous chemicals, and therefore is more friendly to the analytical chemists and to the environment than previous methods.

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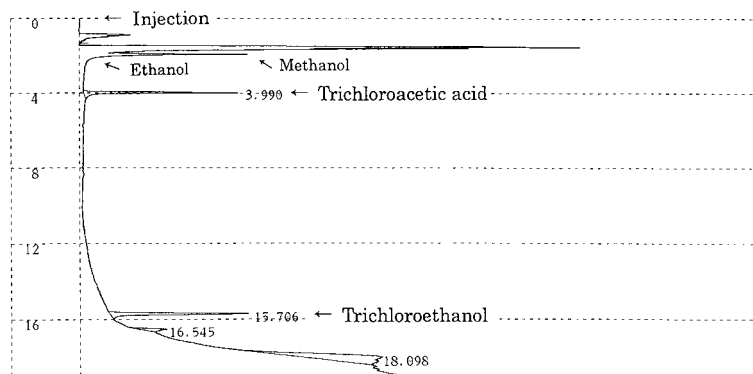


Fig. 1. A typical chromatogram to measure trichloroacetic acid and trichloroethanol.

A urine sample from a worker exposed to trichloroethylene at 19.8 ppm (as an 8 h-time weighted average) was subjected to ECD-GC analysis. The peak of trichloroacetic acid (62.5 mg/l) and trichloroethanol (58.1 mg/l) were detected at 3.99 and 15.71 min, respectively, after injection. Peaks of methanol (at 2.1 min) and ethanol (at 2.3 min as a very small shoulder peak), both employed as components of a diluent in the pretreatment process, also appeared in the earlier part of the chromatogram together with a large peak for un-identified components in urine.

Materials and Methods

Ethical issue

The Ethics Committee of Tohoku Rosai Hospital approved the study protocol, and each of the participants provided his/her informed consent.

Reagents

Authentic TCA (reagent grade) was purchased from Junsei Chemicals, Tokyo, Japan, and was dissolved at a concentration of about 3 g/l for use; the exact concentration was determined by titration. Authentic TCE (reagent grade; 98% in purity) was purchased from Tokyo Kasei Chemicals, Tokyo, Japan. Ethanol (purity, 99.8%) and methanol (purity, 99.5%) were purchased from Junsei Chemicals, Tokyo, Japan, and Wako Pure Chemicals, Osaka, Japan, respectively.

Urine samples

Urine samples for development and validation of the present method were obtained from 19 TRI-exposed workers [12 men and 7 women, exposed to TRI up to 46.4 ppm as the 8-h time-weighted average (TWA) concentration (Table 1)] and 8 non-exposed subjects (5 men and 3 women). Spot urine samples were collected at the end of an 8-h work-shift on Thursday or Friday of a working week. The TWA TRI concentration was measured by diffusive sampling as previously described^{20, 21}.

Pretreatment of urine samples

An aliquot (50 μ l) of concentrated hydrochloric acid (about 36%) was added to 1 ml urine sample from an exposed subject

(or authentic TCA or TCE dissolved in urine from a non-exposed subject) in a tube. In preparing the calibration curves, it is important to use urine samples from non-exposed subjects (and not water) in place of the samples from the exposed; the use of water in place of urine results in a substantially smaller peak (for TCA) than that with urine, suggesting that some unidentified components in urine may influence the determination of TCA although not of TCE.

After the tube was tightly sealed and the contents were well mixed, the mixture was heated at 100°C for 30 min, and spun after cooling for 1 min at 1,600 \times g. A portion (50 μ l) of the supernatant phase was transferred to a tube containing a mixture (3 ml) of ethanol (EtOH), methanol (MeOH) and redistilled water (1: 1: 1 by volume) and thoroughly mixed, and an aliquot (usually 1 μ l, and up to 2 μ l when necessary) was taken to inject into the gas-chromatographic system.

ECD-GC analysis developed in the present study

An electron capture detector-equipped gas-chromatograph (ECD-GC; GC 14A, Shimadzu, Kyoto, Japan) connected with a chromato-processor (C-R4A Chromatopac, Shimadzu, Kyoto, Japan) was employed. The column used was a Neutra Bond-1 capillary column, which was 60 m in length, 0.53 mm in inner diameter and 2.0 μ m in film thickness (G-L Science, Tokyo, Japan). Extra-pure N₂ as carrier gas was allowed to flow to the column at a rate of 17 ml/min. The injection port and the detectors were kept at 280°C. The temperature of the column was maintained at 40°C for 10 min after the injection, and elevated at a rate of 10°C/min to 100°C and then at 40°C/min to 200°C to stay at 200°C

for 0.6 min before cooled down. Under standard conditions, the peaks of TCA and TCE appeared at 4.0 and 15.7 min after the injection, respectively. GC analysis of one sample was completed in 29 min excluding the time for cooling down of the column. A typical chromatogram of a urine sample from a TRI-exposed worker is shown in Fig. 1. Total trichloro-compounds (TTC) were calculated by the addition of TCA and TCE (the method A).

Other methods of TCE and TCA analyses used for comparison

The colorimetry utilizing color formation by alkali pyridine reaction (Fujiwara reaction) was conducted after Tanaka and Ikeda²⁾ (to be called the method B). TTC and TCA were measured with or without oxidative conversion (by chromium trioxide and nitric acid) of TCE to TCA including hydrolysis of conjugated TCE, and TCE was calculated by subtraction of TCA from TTC. The head-space ECD-GC method with hydrolysis of conjugated TCE and derivatization of TCA to its methyl ester was after Ohara *et al.*¹⁾ in which methanol and sulfuric acid were used as the esterification reagent (to be called method C).

Statistical analysis

In cases when the effects of different urine density was considered, the analyte concentration were corrected for creatinine concentration²²⁾ or urine specific gravity of 1.016²³⁾.

As to be described later, the distribution of the analyte concentrations was skewed although the distribution pattern was unknown. Accordingly, non-parametric Wilcoxon signed ranks test was employed for examination of statistical significance of the difference in addition to parametric paired *t*-test. Possible statistical significance of the differences in intercepts and slopes of two regression lines was examined after Ichihara²⁴⁾.

Results

Development of pretreatment conditions

Hydrochloric acid rather than sulfuric acid was preferred for acid hydrolysis of conjugated forms of metabolites (especially TCE) because of higher volatility and less corrosiveness. The hydrolysis condition was considered sufficient based on the observation that the same amounts of TCE were detected in urine samples of workers by the colorimetric method²⁾ and by the present method, as to be described later.

The hydrolyzed samples were thick, and it was necessary to dilute hydrolyzates with an appropriate diluent before GC analysis; high sensitivity of the ECD allowed the dilution of the samples. Whereas the use of water was thought essential to keep TCA dissolved, the performance of a micro-syringe (for injecting 1–2 μ l) was disturbed by friction between the piston and the syringe wall, when pure water

was employed as the sole diluent. The use of EtOH or MeOH alone gave a large peak with a substantially long tail, which disturbed quantification of TCA (with a relatively short retention time of 4.0 min).

Thus, the 1: 1: 1 (by volume) mixture of EtOH: MeOH: water (1 ml, 1 ml and 1 ml in practice) was empirically identified as the diluent of choice. In the pretreatment process, a 50 μ l aliquot of 36% hydrochloric acid was added to 1 ml urine for hydrolysis, and a 50 μ l portion of the hydrolyzate was diluted with 3 ml of the mixture. As a result, the concentration of hydrogen chloride after all dilution steps was <0.03%. Experiences with repeated injections of the pretreated samples to the ECD-GC appeared to suggest that the applications did not induce any damage to the analysis system.

Accuracy and precision of the method

When solutions of authentic TCA (75 and 150 mg/l) and TCE (50 and 100 mg/l) in urine from the non-exposed were repeatedly analyzed five times, the coefficients of variation were 4.5, 3.6, 4.4 and 3.4%, respectively. The recovery rates for TCA (75 and 150 mg/l) and TCE (50 and 100 mg/l) added to 5 urine samples from non-exposed subjects were 100.2 to 101.8% and 99.5 to 99.8%, respectively, as compared with the amounts dissolved in redistilled water. Linear regression analysis taking 0, 75 and 150 mg/l TCA, or 0, 50 and 100 mg/l TCE on the horizontal axis and height of corresponding peaks (peak height or PH) on the vertical axis gave regression lines of: $PH = -0.022 + 21,000 \times TCA$ (mg/l) ($n=15$, $r=0.998$, $p<0.01$) in case of TCA, and $PH = -0.236 + 490,000 \times TCE$ (mg/l) ($n=15$, $r=0.999$, $p<0.01$), for TCE. The lower limits of detection (LOD) were 0.1 mg/l for TCA and 0.05 mg/l for TCE, when a peak/noise ratio of 2 was taken.

Need of urine samples for TCA determination

It was experienced in the course of calibration curve preparation that the peak space for TCA (but not TCE) was substantially (usually by 29%) smaller when water (50 μ l) was added to the diluent mixture in place of urine or urine hydrolysates. Whereas it was not possible to clarify the reason for the difference in the results between water and urine, experiments with 15 urine samples (from the non-exposed) with various specific gravities (from 1.004 to 1.025) showed no effects of the difference in urine density on the peak space for TCA (equivalent to 49.2 mg TCA/l urine). The slope of regression line with the urine density and the specific gravity on the horizontal and the vertical axis was in fact slightly negative (i.e., below zero). The estimated TCA concentration for urine samples with 1.005 as a specific gravity was minimally greater (by <0.5%) than the TCA when urine with a specific gravity of 1.025 was employed. It should be added that no difference was observed between water and urine for TCE determination.

Table 1. Distribution of analytes

Analyte	Unit	Method ^a	AM	ASD	Median	Min.	Max.	p ^b for difference between		
								A and B	B and C	C and A
TRI in air	ppm		13.1	14.0	8.8	0	46.4			
TCA in urine	mg/l	A	9.5	14.1	4.4	0	62.5	ns	ns	ns
	mg/l	B	9.3	13.4	4.3	0	53.8			
	mg/l	C	9.0	16.0	2.0	0	66.1			
TCE in urine	mg/l	A	15.0	21.1	4.4	0	67.9	ns	ns	<0.05
	mg/l	B	15.9	23.2	3.0	0	72.5			
	mg/l	C	15.9	22.4	3.9	0	74.0			
TTC in urine	mg/l	A	24.5	32.9	7.3	0	120.6	ns	ns	ns
	mg/l	B	25.2	34.4	7.0	0	111.5			
	mg/l	C	25.0	35.8	4.8	0	125.8			

In total, 27 cases of both sexes (19 exposed and 8 non-exposed) were analyzed. ^aMethod of determination: A, the present method; B, Tanaka and Ikeda (1968)²; C, Ohara *et al.* (1991)¹. ^bp value by Wilcoxon signed ranks test: the difference in the paired results by the two methods shown; ns for p>0.10.

Comparison of the results as determined by the three methods

The results by the present method (data by the method A) with that by the colorimetric method² (data by the method B) and by the esterification-GC method after Ohara *et al.*¹ (data by the method C) are given in Table 1 in terms of the three target analytes of TCA, TCE and TTC.

It was clear that ASD was larger than the corresponding AM. Comparison by non-parametric Wilcoxon signed ranks test for paired data showed that the difference among the results by the three methods were generally insignificant with one exception that TCE appeared to be different (p<0.05) when measured by the method A and by the method C. Comparison by paired *t*-test after logarithmic conversion gave essentially the same results.

Correlation of the results of analyses by the three methods

The agreement/disagreement in the analysis results among the three methods were also examined by regress analysis (Table 2). For this purpose, the method developed earlier in time was taken as an independent variable, and the later one as a dependent variable. It was clear that the correlation between the paired values were very close with $r>0.95$. The slopes were nearly equal to 1 with the lowest value of 0.863 for the comparison of the method C with the method A for TCA. This may be a reflection of the trends that TCA by the method C tended to give slightly higher value than the method B (with the slope of 1.168 on the 2nd line in the table; also Ohara *et al.*¹), whereas the methods B and A gave essentially the same values for TCA (the top line in the table). The intercepts should be taken essentially zero, knowing that TCA, TCE and TTC were in ranges up to 50 to 100 mg/l. For visual understanding of the cases, the relationships between TTC measured by the three methods

Table 2. Correlation of urinary analyte levels as measured by the three methods

Analyte	Method for the axis of		Regression line parameters		
	X ^a	Y ^a	α^b	β^b	r^c
TCA	B	A	0.05	1.008	0.958
	B	C	-1.877	1.168	0.979
	C	A	1.669	0.863	0.978
TCE	B	A	0.668	0.901	0.992
	B	C	0.746	0.954	0.990
	C	A	0.068	0.938	0.995
TTC	B	A	0.551	0.947	0.992
	B	C	-1.137	1.033	0.994
	C	A	1.647	0.914	0.995

In total, 27 cases of both sexes (19 exposed and 8 non-exposed) were analyzed. ^aFor example, B and A on the columns for X and Y, respectively, mean that the results with method A and B are given on the X and Y axis, respectively. TCA, TCE and TTC are in mg/l. ^b α and β are parameters of calculated regression lines, $Y = \alpha + \beta X$, where X and Y are as given in the table. ^cAll correlation coefficients (r 's) are statistically significant (p<0.01).

are depicted in Fig. 2 as examples, together with calculated regression lines.

Correlation between TRI in air and the urinary metabolite levels as measured by the three methods

The purpose of the urinalysis is to estimate the average intensity of exposure to TRI in workroom air, in addition to the detection of potential exposure via skin. Thus, the correlations were examined between TWA TRI and TCA, TCE or TTC in urine as measured by the three methods. As summarized in Table 3, the correlation with TRI in air was

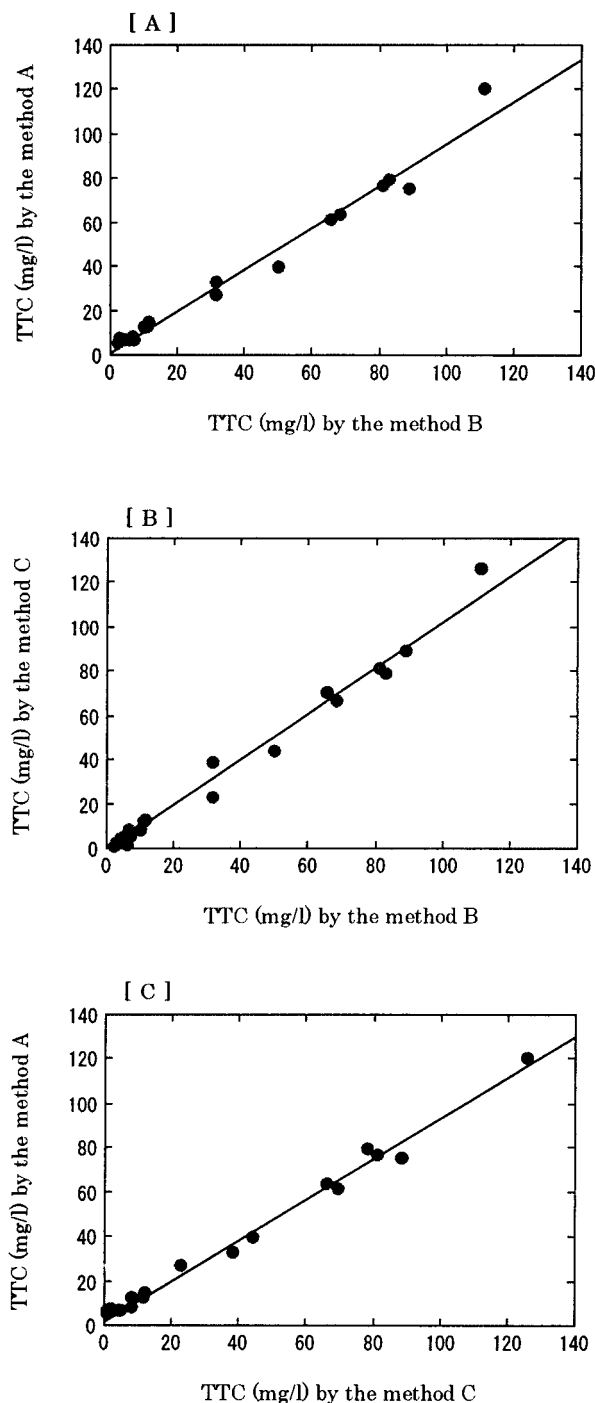


Fig. 2. Quantitative relation between total trichloro-compounds (TTC) as measured by the three methods: [A] the method B vs. the method A, [B] the method B vs. the method C, and [C] the method C vs. the method A.

The methods A, B and C stand for the present method, the colorimetric method after Tanaka and Ikeda²⁾, and the method after Ohara *et al.*¹⁾, respectively. Each dot represents one case (with some over-lapping near the origin), and the lines in the middle are calculated regression lines, the equations for which are given in the three lines for TTC in Table 2. The slope is very close to 1, and the line passes very close to the origin.

close and statistically significant ($p < 0.01$) irrespective of TCA and TCE, and therefore TTC, although the correlation coefficients (r 's) tended to be greater for TCE (> 0.90) than for TCA (< 0.60 ; P for the difference was about 0.10, as examined by Wilcoxon signed ranks test). Nevertheless, the intercepts and slopes did not differ significantly ($p > 0.10$; after Ichikawa²⁴⁾). In other words, the three methods give the same results when applied to urinalysis for estimation of intensity of exposure to TRI.

Possible effects of correction for urine density

As the effects of correction of analyte levels for urine density has been a focus of long-standing discussion (e.g., Moriguchi *et al.*^{25,26)}), the present data set by the method A was subjected to analysis for possible improvement in relationship of the analyte levels with TRI exposure (Table 4). Thus, the correlation with TWA TRI was compared after no correction ('none' in the table), correction for creatinine and that for specific gravity taking 1.016 as a standard. It was clear that the correlation was significant ($p < 0.01$ except for one case, for which p was < 0.05) for all cases. It appeared also likely that the correlation was closer for TCE ($r = 0.87$ to 0.90) than for TCA ($r = 0.46$ to 0.55) ($p = 0.10$ by Wilcoxon signed ranks text). Further comparison among the three correction conditions showed that the values without correction (i.e., the observed values) tended to give the highest correlation followed by the values corrected for specific gravity and the values corrected for creatinine concentration gave the lowest one ($p = 0.10$ by Wilcoxon signed ranks text). Thus, contrary to expectation, correction for urine density did not induce any improvement in the correction.

No need of acid treatment for TCA determination

Heat treatment of the urine samples in presence of hydrochloric acid was primarily for hydrolysis of conjugated TCE. To examine if it is possible to determine TCA without the acid treatment, comparative analyses were conducted with a urine sample from TRI exposed worker under three conditions, i.e., 1. as developed in the present study, 2. with no acid or heat treatment (with due adjustment of the total volume with re-distilled water), and 3. with acid addition but no heat treatment. The analyses were repeated by 5 times each. The samples thus treated ($50 \mu\text{l}$) were diluted with a 3 ml portion of an EtOH: MeOH: water mixture as described before, and were subjected to the ECD-GC analysis. The average TCA concentrations detected under three conditions were 61.8, 61.7 and 61.6 mg/l with CV of about 3%. The results showed that TCA determination is not affected by the presence or absence of the acid for hydrolysis.

Discussion

As described in the Introduction section, urinalysis for

TCA, TCE or both are popular in biological monitoring of exposure to TRI, TETRA and MC in occupational health service for workers exposed to the chlorinated hydrocarbon solvents. In Japan, for example, the urinalysis is among the legal requirements²⁷⁾.

Despite such common use, the urinalysis needs the use of potentially hazardous chemicals. Colorimetric determination, historically used in industrial health for many years^{2, 15)}, uses chromium trioxide (a Group 1 carcinogen as a hexa-valent chromium, according to the classification of International Agency for Research on Cancer²⁸⁾) in

combination with concentrated nitric acid for hydrolysis of conjugated TCE conjugates and oxidative conversion of liberated TCE to TCA, and potassium hydroxide (caustic) and pyridine (with objectionable smell) for color-generating Fujiwara reaction. Head-space gaschromatography with an electron-capture detector (HS-GC), which was subsequently developed, also needs potentially hazardous dimethyl sulfate, diazomethane or a mixture of methanol and sulfuric acid for conversion of non-volatile TCA to volatile TCA methyl ester by esterification¹⁾. The two methylating reagents, dimethyl sulfate (Group 2A carcinogen with sufficient evidence on experimental animals and limited evidence on humans²⁹⁾) and diazomethane (Group 3 with limited evidence on experimental animals and no adequate data on humans^{30, 31)}) are carcinogenic at least to animals.

The use of carcinogens or objectionable reagents is very contrary to the latest trends in chemistry, i.e., green chemistry or sustainable chemistry (or the chemistry friendly to man and the environment³²⁻³⁴⁾). The efforts for green chemistry have been made not only in chemical industry activities³⁴⁻³⁶⁾, but also in analytical chemistry^{37, 38)}. The method developed in the present study needs only low molecular weight alcohols (MeOH and EtOH) and water for analysis of TCA, and a minute amount of HCl in addition for hydrolysis of conjugated TCE. The method is thus friendly to the chemists engaged in the analysis, and certainly to the environment as well.

The LOD of the present method is 0.1 mg/l for TCA and 0.05 mg for TCE. The LODs are low enough for biological monitoring of occupationally exposed workers. The expected levels of TCA and TCA+TCE (i.e., TTC) after occupational exposure to TRI at the occupational exposure limit of 25 ppm are 50 and 150 mg/l urine³⁹⁾. Another organization

Table 3. Correlation of urinary analyte levels by the three methods with TRI in air

Y	Method	α^a	β^b	r^b
TCA	A	2.272	0.551	0.546
	B	2.119	0.553	0.577
	C	1.154	0.603	0.527
TCE	A	-2.817	1.364	0.904
	B	-3.835	1.511	0.910
	C	-3.059	1.453	0.908
TTC	A	-0.545	1.915	0.814
	B	-1.716	2.064	0.838
	C	-1.905	2.056	0.803

In total, 27 cases of both sexes (19 exposed and 8 non-exposed) were analyzed. ^a α and β are parameters of calculated regression lines, $Y = \alpha + \beta X$, where X is TRI in air (ppm) and Y is the analytes in urine (mg/l) as shown in the table. There were no significant differences ($p > 0.10$) among the intercepts as well as slopes of the regression lines by the three methods. ^bAll correlation coefficients (r 's) are statistically significant ($p < 0.01$).

Table 4. Possible effects of urine density correction on correlation with TRI exposure

Y (Analyte)	Correction for urine density	α^a	β^b	r^b
TCA	None	2.272	0.551	0.546**
	Creatinine	3.458	0.312	0.460*
	Specific gravity	2.513	0.355	0.512**
TCE	None	-2.817	1.364	0.904**
	Creatinine	-0.371	0.867	0.870**
	Specific gravity	-1.098	0.904	0.886**
TTC	None	-0.545	1.915	0.814**
	Creatinine	3.087	1.179	0.770**
	Specific gravity	1.415	1.259	0.786**

In total, 27 cases of both sexes (19 exposed and 8 non-exposed) were analyzed. ^a α and β are parameters of calculated regression lines, $Y = \alpha + \beta X$, where X is TRI in air (ppm) and Y is the analytes in urine (mg/l for non-corrected or specific gravity-corrected values, and mg/g creatinine for creatinine-corrected values) as shown in the table. 1.016 was taken as a standard specific gravity. ^bAll correlation coefficients (r 's) are statistically significant (** and * for $p < 0.01$ and < 0.05 , respectively).

gives 100 and 300 mg/g creatinine after exposure at the threshold limit value of 50 ppm⁴⁰). The sensitivity is apparently insufficient, however, for monitoring TCA levels in urine of general populations who are exposed to disinfection by-products in chlorination-treated drinking water^{41–45}. Monitoring the expected TCA levels of 0.5 to 25 µg/l urine needs much more sophisticated expensive methods such as isotope-dilution HPLC-tandem mass spectrometry^{43, 44}.

No need of acid or heat treatment for determination of TCA alone is advantageous for practical application. Above all, the deletion of hand-consuming procedure of acid hydrolysis is important, because a urine sample can be analyzed by ECD-GC for TCA just after dilution with a EtOH: MeOH: water mixture. From toxicological viewpoints, the urinary excretion of TCA has a longer biological half-life than that of TCE in TRI-exposed workers (i.e., about 40–58 h for TCA versus about 15–43 h for TCE¹⁴) so that TCE levels in urine reflect TRI exposure of the day more closely than TCA (as shown in Tables 3 and 4), whereas TCA levels may show the average exposure over a longer term. TCA was a major urinary metabolite in case of exposure to tetrachloroethylene (TETRA)^{11–13} with a half-life of 120–190 h¹⁴). In other words, TCA may be a better indicator of long-term TRI exposure and a urinary biomarker of choice to monitor exposure to TETRA. In this sense, simplified procedure for TCA determination is of practical importance.

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