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Development of Simultaneous HPLC-Fluorescence Assay of Phenol and Chlorophenols in Tap Water after Pre-Column Derivatization with 3-Chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone

Yasuhiko Higashi

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Japan
Email: y-higashi@hokuriku-u.ac.jp

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Abstract

Chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol and 2,4,6-trichlorophenol) may be presented in natural waters or drinking water as a result of disinfection processes involving chlorination, or as contaminants derived from domestic products, industrial operations and agricultural chemicals. A previous HPLC-UV method for determination of phenol and five chlorophenols in tap water using 4-fluoro-7-nitro-2,1,3-benzoxadiaole as a UV labeling reagent shows limited sensitivity. Here, we present an improved HPLC-fluorescence detection method for simultaneous determination of phenol and the above chlorophenols in tap water after pre-column derivatization with 3-chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (DMEQ-COCl), using a short, narrow column (50 × 2.1 mm i.d., packed with 5 μm particles of C_{18} material) to improve the sensitivity. Standard samples containing the compounds are derivatized with DMEQ-COCl in borate buffer (pH 9.0) at room temperature for 3 mins. The response is linear in the concentration range of 0.01 - 0.05 to 0.5 mg/L with r^2 values ≥0.9967 for all compounds. The lower limits of detection are 0.001 to 0.008 mg/L, and the coefficients of variation are less than 8.8%. The recovery values from tap water spiked with standard samples are satisfactory. The present method is suitable for examining whether or not tap water samples are contaminated with phenol and chlorophenols in excess of regulatory values.

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Keywords

Phenol, Chlorophenol, Fluorescence, Derivatization, 3-Chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone

1. Introduction

In order to confirm the quality and safety of potable water, it is essential to evaluate levels of pollutants. Phenol is a ubiquitous pollutant in the aquatic environment because of its widespread use in the synthesis of dyes and drugs in the chemical industry and its presence in commercial products used in daily life. Five chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, and 2,4,6-trichlorophenol) are also presented in drinking water as a result of disinfection by means of chlorination, as well as being formed by the reaction of hypochlorite (used as a bleach and disinfectant) with phenolic acids and via the degradation of phenoxy herbicides [1].

The World Health Organization (WHO) guideline value for 2,4,6-trichlorophenol is 0.2 mg/L and concentrations of chlorophenols in drinking-water are usually less than 0.001 mg/L [1]. The maximum permissible level of total phenols is 0.5 mg/L in drinking water, but concentration of individual phenols must not exceed 0.1 mg/L according to United States Environmental Protection and European Union guidelines [2]-[4]. On the other hand, the maximum permissible level of total phenols according to the Japanese Water Pollution Control Law is less than 0.005 mg/L in drinking and tap water and less than 5 mg/L in industrial waste water. A widely used analytical method for determination of total phenols in water samples is visible absorbance measurement after reaction with 4-aminoantipyrine [5] [6]. However, this method cannot determine the concentrations of individual phenols. Various separation methods for phenols have been reported, employing GC, HPLC, and capillary electrophoresis with several detection modes, such as fluorimetry, mass spectrometry, chemiluminescence, and electrochemical analysis [7]-[12].

Derivatization with a UV absorber or a fluorescent compound that can react with a functional group of the target compound(s) is one of the most useful strategies to obtain high selectivity and sensitivity. Phenol in river water and wine has been determined by means of HPLC-UV after derivatization with benzoyl chloride, and simultaneous analysis of phenol and chlorophenols in urine has been achieved by means of HPLC with fluorescence detection (FLD) after derivatization with 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride, though this method requires sample pretreatment [4] [13]. There is also a report describing HPLC-fluorescence detection after derivatization with coumarin-6-sulfonyl chloride for determining phenol and chlorophenols in various environmental waters, with sample clean-up by means of filtration [10].

Since it is very important to quickly check and confirm the quality of water throughout the world, we set out to develop a sensitive and selective method for simultaneous determination of phenol and chlorophenols without any clean-up step. 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) has been used for HPLC-FLD as a fluorescent label reactive with primary and secondary amino groups [14]-[18]. Toyo'oka *et al*. also used NBD-F as a UV labeling reagent for the phenolic hydroxyl group of *N*-acetyltyrosine [19]. Subsequently, an HPLC-UV determination of phenol and five chlorophenols is developed using NBD-F for the analysis of tap water without sample clean-up [20], but its sensitivity is limited (0.004 to 0.1 mg/L).

Here, we present a sensitive HPLC-FLD method for simultaneous determination of phenol and five chlorophenols in tap water after pre-column derivatization with 3-chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (DMEQ-COCl; **Figure 1**), without the need for sample clean-up. The use of a narrow, short analysis column is helpful to increase peak sharpness, which contributes to improving the sensitivity.

2. Materials and Methods

2.1. Materials

Phenol, 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol, DMEQ-COCl, and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Tap water was collected from the laboratory supply.

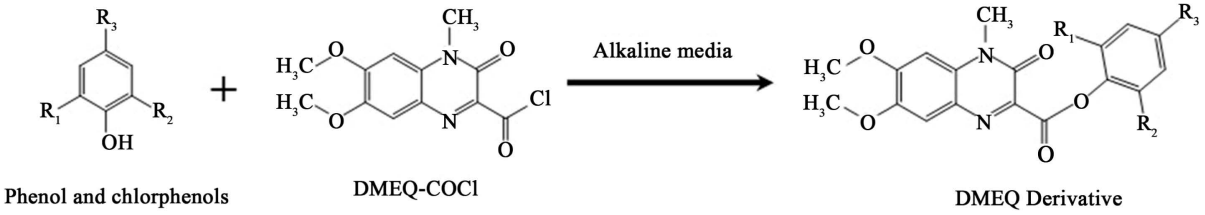

Figure 1. Derivatization of phenol and chlorophenols with DMEQ-COCl. Phenol: $R_1 = R_2 = R_3 = H$; 2-Chlorophenol: $R_1 = Cl$, $R_2 = R_3 = H$; 4-Chlorophenol: $R_1 = R_2 = H$, $R_3 = Cl$; 2,4-Dichlorophenol: $R_1 = R_2 = Cl$, $R_3 = H$; 2,6-Dichlorophenol: $R_1 = R_3 = Cl$, $R_2 = H$; 2,4,6-Trichlorophenol: $R_1 = R_2 = R_3 = Cl$.

2.2. Chromatographic Conditions

The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne injection valve (Cotati. CA, USA) with a 20-µL loop, and a model RF-10A_{XL} fluorescence detector (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 400 nm and emission wavelength of 500 nm. The HPLC column (Inertsil ODS-4, GL Science Inc., Kyoto) was 50 × 2.1 mm i.d., packed with 5 µm particles of C_{18} material. Quantification of the peaks was performed using a Chromatopac Model C-R3A integrator (Shimadzu). The mobile phase was prepared by the addition of acetonitrile (320 mL) to a solution of 680 mL containing trifluoroacetic acid (0.1 v/v%) in ultrapure water (Milli-Q water purification system, Simplicity® UV, Millipore Corporation, Bedford, MA, USA). The samples were eluted from the column at room temperature at a flow rate of 0.5 mL/min.

2.3. Derivatization

Standard samples of phenol and chlorophenols were dissolved in ultrapure water and acetone, respectively, and adjusted to the concentration of 1 g/L. The standard mixture was diluted with ultrapure water. Borate buffer (0.1 M) was adjusted to pH 9.0 by the addition of NaOH. Borate buffer (100 µL) was added to diluted standard samples (100 µL, containing 0, 0.01, 0.02, 0.05, 0.1, 0.2, or 0.5 mg/L of each compound). DMEQ-COCl solution in acetonitrile (2 mg/mL, 100 µL) was added and vortexed. The mixture was allowed to react for 3 min at room temperature. Saturated L-aspartate solution (100 µL) was added to stop the reaction, and aliquots (10 µL) were injected into the HPLC system.

2.4. Application to Water Samples

Water samples spiked with standard samples (lower limit of quantification, 0.05, 0.1, 0.2, or 0.5 mg/L of each compound) were passed through 0.45 µm filters (Cosmonice Filter S, Nacalaitesque) to remove suspended substances and immediately analyzed. Filtration was confirmed to have no effect on the analysis by analyzing a sample spiked with standards before and after filtration; no significant difference in recovery was observed.

2.5. Relative Recovery

Relative recovery was expressed as the ratio of the slope of the calibration curve prepared from a water sample spiked with standard sample to the slope of the standard calibration curve prepared as described above. Relative recovery data were used to assess the accuracy of the method.

3. Results and Discussion

3.1. Derivatization of Phenol and Five Chlorophenols with DMEQ-COCl

For the time course study, the reaction time was set at 2, 3, 5, 10, and 15 min. Phenol and five chlorophenols (100 µL, each 0.1 mg/L), borate buffer (100 µL, pH 9.0)and DMEQ-COCl (100 µL, 2 mg/mL) were mixed as described in Materials and Methods. The maximum peak area or plateau level was reached within 3 mins (**Figure 2**).

Next, pH dependency (pH 7.5 to 10.0) was examined at the derivatization time of 3 min (**Figure 3**). Peak areas of DMEQ-phenol and DMEQ-4-chlorophenol at pH 7.5 were 33% and 82% of the maximum area, respectively. The peak areas of all DMEQ derivatives tended to be decreased at pH 10.0. However, the peak areas of

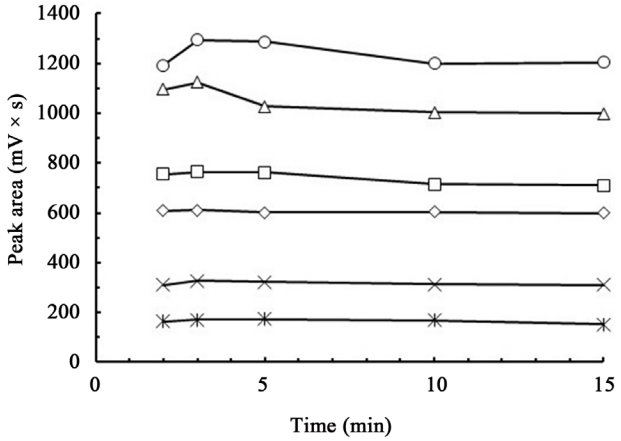

Figure 2. Time courses of formation of the derivatives of phenol and chlorophenols with DMEQ-COCl. Standard sample (containing each compound 0.1 mg/L) was allowed to react with DMEQ-COCl in borate buffer at pH 9.0. (○), DMEQ-phenol; (△), DMEQ-4-chlorophenol; (□), DMEQ-2-chlorophenol; (◇), DMEQ-2,4-dichlorophenol; (×), DMEQ-2,6-dichlorophenol; (*), DMEQ-2,4,6-trichlorophenol.

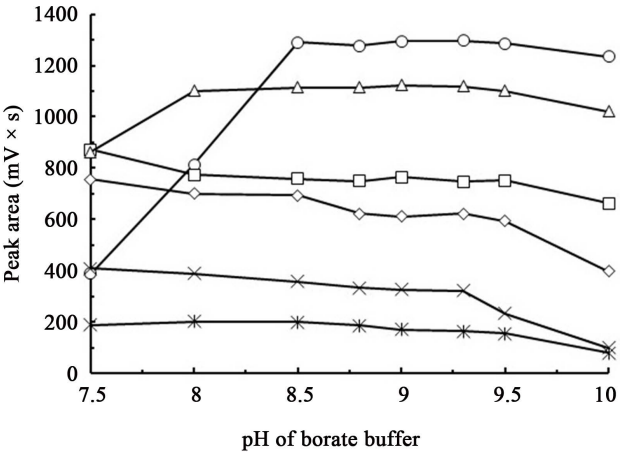

Figure 3. pH Dependency of formation of the derivatives of phenol and chlorophenols with DMEQ-COCl. Standard sample (containing each compound 0.1 mg/L) was allowed to react with DMEQ-COCl in borate buffer at various pH values. (○), DMEQ-phenol; (△), DMEQ-4-chlorophenol; (□), DMEQ-2-chlorophenol; (◇), DMEQ-2,4-dichlorophenol; (×), DMEQ-2,6-dichlorophenol; (*), DMEQ-2,4,6-trichlorophenol.

all compounds were stable (more than 95% of the maximum area) in the range of pH 8.5 to pH 9.3.Since, under pH 8.5, it is guessed that a little pH change may effect phenol detection, simultaneous determination of tested compounds is not better at pH 8.5.

In preliminary tests (data not shown), the peak areas of all derivatives obtained with 1 mg/mL of DMEQ-COCl at pH 9.0 and room temperature were less than about 60% of the control. Furthermore, when using 3 mg/mL of DMEQ-COCl, a large blank peak interfered with the peaks of phenol, 2-chlorophenol, and 4-chlo−rophenol. Thus, the derivatization time of 3 min at pH 9.0using 2 mg/mL of DMEQ-COCl was chosen for the assay.

3.2. Chromatogram

Figure 4 shows typical chromatograms obtained from (A) blank and (B) standard sample (0.1 mg/L). In the analysis, full scale (128 mV) was automatically shifted to 64 mV at 20 min to clearly observe the DMEQ-2,4,6-trichlorophenol peak. The retention times of DMEQ-phenol, DMEQ-2-chlorophenol, DMEQ-4-chlorophenol,

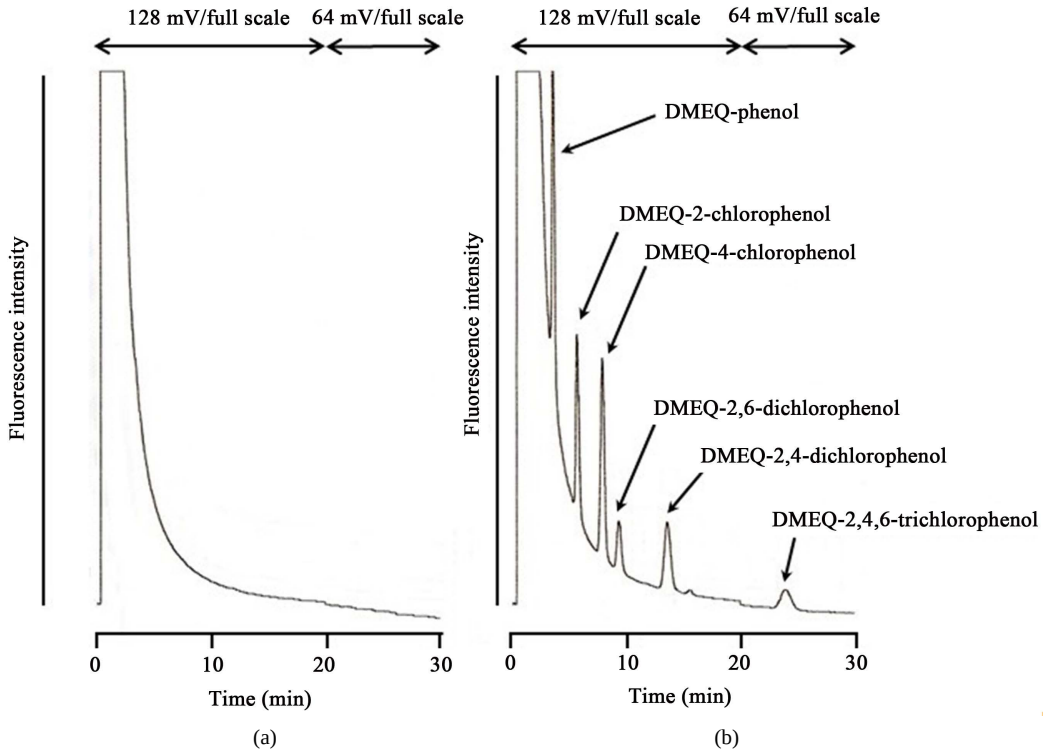

Figure 4. Typical chromatograms of blank (a) and standard samples (b) after derivatization with DMEQ-COCl. Standard sample (containing each compound 0.1 mg/L) was allowed to react with DMEQ-COCl for 3 mins at pH 9.0. Retention times (min): 3.6 DMEQ-phenol; 5.7, DMEQ-2-chlorophenol; 7.9, DMEQ-4-chlorophenol; 9.3, DMEQ-2,6-dichlorophenol; 13.6, DMEQ-2,4-dichlorophenol; 23.9, DMEQ-2,4,6-trichlorophenol. Fill scale was automatically shifted to 64 mV at 20 mins.

DMEQ-2,6-dichlorophenol, DMEQ-2,4-dichlorophenol, and DMEQ-2,4,6-trichlorophenol were 3.6, 5.7, 7.9, 9.3, 13.6, and 23.9 min, respectively. The running time was 30 mins.

3.3. Standard Curves of Phenol and Five Chlorophenols

The standard curves of phenol, 2-chlorophenol, 4-chlorophenol, 2,6-dichlorophenol, 2,4-dichlorophenol, and 2,4,6-trichlorophenol were constructed by plotting integrated peak areas of derivatives *vs.* concentrations of phenolic compounds. The calibration data are summarized in **Table 1**. The plots were linear in the concentration range of 0.01 - 0.05 to 0.5 mg/L, with r^2 value $\geq$ 0.9967 for all compounds. The values of the lower limit of quantification were taken as the lowest concentration on the standard curve. The lower limits of detection for phenol and 2-chlorophenol were estimated as the concentrations giving a detectable peak, since these peaks were located close to a large blank peak. The lower limits of detection for 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, and 2,4,6-trichlorophenol were taken to be the concentrations giving a signal-to-noise ratio of 3:1. The lower limits of detection for phenol, 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, and 2,4,6-trichlorophenol were 0.003, 0.002, 0.001, 0.004, 0.002, and 0.008 mg/L, respectively (range of 0.0025 to 0.020 ng/20 μL injection as absolute amount).

The method using NBD-F previously developed in our laboratory showed relatively poor sensitivity [20]. The present method shows 1.2- to 5-fold greater sensitivity in terms of the lower limits of detection (8.5- to 33-fold improvement in terms of absolute amount).

3.4. Precision and Accuracy

Precision and accuracy for intra-day and inter-day assays of these derivatives are shown in **Table 2** and **Table 3**. In the intra-day assay, the range of standard deviation was within 2.7% to 8.0%. The recoveries were within 96.5% to 104.2%. In the inter-day assay, the range of standard deviation was within 4.3% to 8.9%, and the recoveries were within 95.5% to 105.0%.

Table 1. Linear correlation parameters.

Compounds	Slope	Intercept	Concentration range	r^2	Lower limit of detection (S/N = 3)
Phenol	12,057	+50.61	0.01 to 0.5 mg/L	0.9995	0.003 mg/L (0.0075*)
2-Chlorophenol	8892	−88.43	0.01 to 0.5 mg/L	0.9982	0.002 mg/L (0.0050*)
4-Chlorophenol	10,422	+43.71	0.01 to 0.5 mg/L	0.9984	0.001 mg/L (0.0025*)
2,6-Dichlorophenol	2799	+41.28	0.02 to 0.5 mg/L	0.9967	0.004 mg/L (0.010*)
2,4-Dichlorophenol	5315	+46.36	0.01 to 0.5 mg/L	0.9990	0.002 mg/L (0.0050*)
2,4,6-Trichlorophenol	1555	+18.95	0.05 to 0.5 mg/L	0.9972	0.008 mg/L (0.020*)

*: Data are expressed as absolute amount (ng/20 μL injection).

Table 2. Intra-day assay reproducibility for determination of phenol and chlorophenols

Compounds (mg/L)	Measured (mg/L, Mean ± S.D., n = 5)	C.V. (%)	Recovery (%)
Phenol			
0.02	0.0193 ± 0.0015	8.0	96.5
0.1	0.0972 ± 0.0052	5.4	97.2
0.5	0.516 ± 0.021	4.1	103.2
2-Chlorophenol			
0.02	0.0204 ± 0.0016	7.8	102.0
0.1	0.0998 ± 0.0064	6.4	99.8
0.5	0.512 ± 0.019	3.7	102.4
4-Chlorophenol			
0.02	0.0198 ± 0.0014	7.0	99.0
0.1	0.102 ± 0.006	5.9	102.0
0.5	0.521 ± 0.020	3.8	104.2
2,6-Chlorophenol			
0.02	0.0198 ± 0.0015	7.6	99.0
0.1	0.0986 ± 0.0043	4.4	98.6
0.5	0.504 ± 0.018	3.6	100.8
2,4-Chlorophenol			
0.02	0.0204 ± 0.0014	6.8	102.0
0.1	0.0984 ± 0.0034	3.5	98.4
0.5	0.512 ± 0.014	2.7	102.4
2,4,6-Trichlorophenol			
0.1	0.102 ± 0.007	6.9	102.0
0.5	0.498 ± 0.015	3.0	99.6

Table 3. Inter-day assay reproducibility for determination of phenol and chlorophenols.

Compounds (mg/L)	Measured (mg/L, Mean ± S.D., n = 5)	C.V. (%)	Recovery (%)
Phenol			
0.02	0.0191 ± 0.0017	8.9	95.5
0.1	0.0966 ± 0.0061	6.3	96.6
0.5	0.525 ± 0.028	5.3	105.0
2-Chlorophenol			
0.02	0.0208 ± 0.0017	8.2	104.0
0.1	0.0972 ± 0.0070	7.2	97.2
0.5	0.482 ± 0.022	4.6	96.4
4-Chlorophenol			
0.02	0.0206 ± 0.0016	7.8	103.0
0.1	0.103 ± 0.007	6.8	103.0
0.5	0.486 ± 0.027	5.6	97.2
2,6-Dichlorophenol			
0.02	0.0198 ± 0.0016	8.1	99.0
0.1	0.0976 ± 0.0052	5.3	97.6
0.5	0.514 ± 0.022	4.3	102.8
2,4-Dichlorophenol			
0.02	0.0208 ± 0.0015	7.2	104.0
0.1	0.0984 ± 0.0065	6.6	98.4
0.5	0.512 ± 0.024	4.7	102.4
2,4,6-Trichlorophenol			
0.1	0.104 ± 0.009	8.7	104.0
0.5	0.493 ± 0.022	4.5	98.6

3.5. Environmental Analysis

Tap water samples were collected from the laboratory, and the proposed method was employed to determine phenol and chlorophenols in spiked tap water. As shown in **Table 4**, phenol and chlorophenols in tap water samples were below the lower limits of quantification in unspiked samples. Calibration curves prepared from tap water samples spiked with the six compounds showed a linear relationship between concentration and peak response, with $r^2 \geq 0.9933$. The slopes of the calibration curves were similar to those of the standard calibration curves, and the relative recovery values were 89.8% to 111.0%. These results indicate that our method is capable of monitoring very low levels of contamination with phenol and chlorophenols in tap water.

4. Conclusion

A simple HPLC-UV method for simultaneous determination of phenol and chlorophenols in water was previously developed using NBD-F or benzoyl chloride as the derivatizing reagent, without complicated sample clean-up [4] [20]. Here, to improve the sensitivity, we developed an HPLC-FLD system (column size; 50 × 2.1 mm i.d.) using DMEQ-COCl as a fluorescence labeling reagent. This method was simple, reproducible, and sufficiently sensitive to establish whether or not tap water was contaminated with phenol, 2-chlorophenol, 4-chloro-

Table 4. Levels of phenol and chlorophenols in tap water, and the relative recovery.

Compounds	Concentration in tap water sample	Relative recovery (%, mean ± S.D., n = 5)	r^2 (Average)
Phenol	N.D.	89.8 ± 6.1	0.9933
2-Chlorophenol	N.D.	92.6 ± 6.6	0.9946
4-Chlorophenol	N.D.	94.2 ± 5.6	0.9971
2,6-Dichlorophenol	N.D.	92.4 ± 6.6	0.9972
2,4-Dichlorophenol	N.D.	106.8 ± 7.4	0.9965
2,4,6-Trichlorophenol	N.D.	111.0 ± 6.9	0.9942

N.D.: not determined.

phenol, 2,4-dichlorophenol, 2,6-dichlorophenol, and 2,4,6-trichlorophenol (*i.e.* whether or not these compounds were present in excess of regulatory levels).

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