

Analysis of Chlorinated Phenols in Animal Organs

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Received 17 September 1999

Chronic latent shortage of natural antioxidants in the diet of experimental animals has been shown to enhance toxic effect of dichlorophenol. For the experimental studies, a GC method for the determination of 2,4-dichlorophenol in organs and blood serum of guinea pigs has been developed. The method is based on hydrolytic splitting of the conjugates prior to isolation of the compound from the organs by distillation with dichloromethane. For isolation from the serum, solid-phase extraction on Empore extraction discs was employed. The recoveries for 2,4-dichlorophenol contents of 0.005 to $10.0 \mu\text{g g}^{-1}$ in the tissues were between 81.2 % and 97.3 %, and between 89.9 % and 95.1 % for the content range of 0.01 to $1.0 \mu\text{g g}^{-1}$ in serum. The LOQ of the method is $5 \times 10^{-4} \mu\text{g g}^{-1}$, the relative standard deviation was below 7 %. The results of the study indicated lower accumulation rate of dichlorophenols in animals fed by higher doses of ascorbic acid.

Increasing occurrence of chlorophenols in the environment and foods, their influence on the ecological balance and toxic effects on lower and higher organisms are currently a global problem. The supply of chlorophenols into the environment grows with intensive use of chemicals in the agriculture and with the development of chemical industry [1–3]. Chlorophenols are reactive compounds possessing biological activity and bio-accumulation capacity [4, 5]. Literature data as well as our studies clearly showed the exposition of general population to chlorinated phenols [6–8].

Most analytical methods for the determination of chlorophenols in biological and environmental samples concern pentachlorophenol, which had been largely used in industry and agriculture. Matrices analyzed are surface and ground water, wood, soil, and biological materials. Very few papers deal with analysis of chlorophenols in foods and in blood [9–13]. Isolation of chlorophenols from liquid matrices (water, blood, urine) is still mostly based on liquid–liquid extraction of acidified samples with organic solvent [14–16]. For solid matrices (soil, foods, biological material), Soxhlet extraction with organic solvents, sonication, or supercritical fluid extraction (SFE) [17, 18] are employed. New techniques of isolation from water and biological samples (urine, blood serum) are based on solid-phase microextraction using phenyl-, octyl-, and octadecylsilica-bonded phases [19–22]. Other procedures make use of the relatively high vapour pressure of chlorophenols which makes it possible to employ simultaneous extraction/distillation with water vapour and organic solvent after splitting the conjugates. Various types of glass apparatus are used for

this work [9–11]. Identification and determination usually rely on gas chromatography on commercially available capillary columns that require derivatization of chlorophenols [10, 24, 25]. Free chlorophenols can be analyzed by HPLC, however, with much lower sensitivity [26].

The objective of the present study was to investigate the protective effects of antioxidants against toxic effects of 2,4-dichlorophenol and, consequently, on lowering the pathologic effects of oxidation which is caused by too low intake of natural antioxidants from food, as well as by severe environmental contamination. Animal experiments showed that chronic shortage of antioxidants in the diet enhances the toxic effect of dichlorophenol. On the other hand, optimization of the ascorbic acid intake decreases the accumulation of 2,4-dichlorophenol in some animal organs.

The compounds studied (2,4-dichlorophenol and its only detectable metabolite 4-monochlorophenol) were analyzed by gas chromatography with EC and MS detection. A distillation method carried out in a special all-glass apparatus has been worked out for isolation of 2,4-dichlorophenol and 4-chlorophenol from liver, kidney, brain, and adipose tissue. For isolation of the compounds from the serum, extraction on Empore Disc Cartridges was employed.

EXPERIMENTAL

Male guinea pigs were given drinking water that provided ascorbic acid (AA) at two doses: 2 mg day^{-1} per animal and 50 mg day^{-1} per animal. After three months, two groups (one of each AA level) were fed by 40 mg of 2,4-dichlorophenol during 2 weeks. After

the termination of the experiment all animals were sacrificed and the organs (liver, kidney, brain, adipose tissue) as well as blood serum were analyzed for 2,4-dichlorophenol and 4-monochlorophenol.

The phenols (Phenols Kit purity 96 %) were purchased from Supelco (Gland, Switzerland). Dichloromethane, toluene, ethanol, methanol, sulfuric acid, hydrochloric acid, sodium hydroxide pellets, kalium hydroxide pellets, anhydrous sodium sulfate were supplied by Merck. Capillary column: PTETM - 5 (30 m \times 0.25 mm, FT 0.25 μ m) (Hewlett—Packard) and 3M Empore high-performance extraction disc cartridges with polystyrene-divinylbenzene copolymer SDB-XC (Varian) were used.

Standard stock solutions of 2,4-dichlorophenol (2,4-DCP), 2,6-dibromophenol (2,6-DBP, internal standard), and 4-monochlorophenol (4-MCP) were prepared in ethanol at concentrations of 100 μ g cm⁻³. From the 2,4-DCP stock solution aqueous solutions were prepared at concentrations of 0.1 μ g cm⁻³, 1.0 μ g cm⁻³, 5.0 μ g cm⁻³, 10.0 μ g cm⁻³, and 20.0 μ g cm⁻³. From the stock solutions of 2,6-DBP and 4-MCP, 10 μ g cm⁻³ solutions were prepared.

Calibration curves were obtained by adding 2,4-DCP standard solutions to 5 g of the liver, kidney, brain, and adipose tissue homogenates from the control group of animals as follows: 0.5 cm³ of 2,4-DCP solution (1.0 μ g cm⁻³), 0.5 cm³ and 1 cm³ (5.0 μ g cm⁻³), 0.75 cm³, 1.0 cm³, 1.25 cm³ (10.0 μ g cm⁻³), and 0.75 cm³, 1.0 cm³, 1.25 cm³ (20.0 μ g cm⁻³). For blood serum analysis, the calibration curves were prepared by adding the following amounts of 2,4-DCP standard solutions to 200 mm³ of the serum: 20 mm³ (0.1 μ g cm⁻³), 10 mm³, 20 mm³, 40 mm³, 60 mm³, 80 mm³, 100 mm³, 140 mm³, and 200 mm³ (1.0 μ g cm⁻³). The control organ and serum samples were occasionally pooled from several animals. Spiked samples as well as the blanks were analytically processed as described above. The calibration curves for the organs were linear within the content range of 0.1 to 5.0 μ g g⁻¹, the calibration curve for serum within 0.01 to 1 μ g cm⁻³ (Figs. 2 and 3). The calibration curves were drawn by plotting the peak areas divided by the peak area of the internal standard 2,6-DBP.

For isolation of chlorophenols from tissues, 50 mm³ of internal standard solution (2,6-DBP, 10 μ g cm⁻³) and 20 cm³ of 30 % ethanolic KOH were added to 1—5 g of tissue homogenate and boiled for 30 min under reflux. After termination of the hydrolytic cleavage of the conjugates, ethanol was evaporated on a rotary vacuum evaporator. 100 cm³ of distilled water were added to the residue, pH was adjusted to 1 with 5 M-H₂SO₄ and 25 cm³ of dichloromethane were added. The flask was then attached to a distillation column equipped with a safety absorber (Fig. 1, "a") filled with 5 cm³ of 0.1 M-NaOH. Isolation was carried out by simultaneous extraction and steam distillation with water vapour and dichloromethane. Organic phase ac-

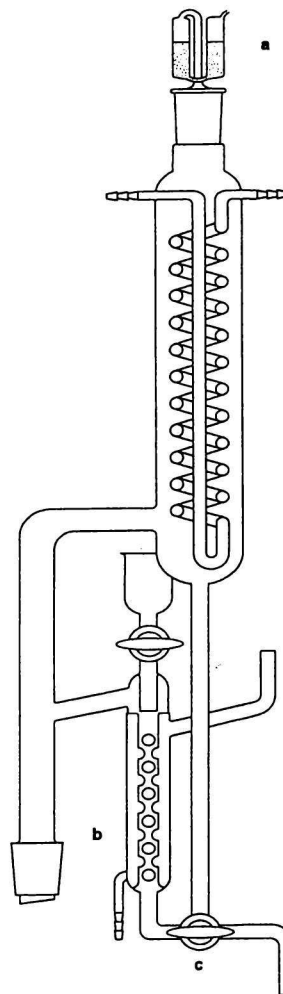


Fig. 1. Modified apparatus for simultaneous distillation and extraction of chlorophenols [9].

cumulated in the lower column (Fig. 1, "b") was transferred into a separatory funnel through a three-way vent (Fig. 1, "c"). The content of the safety absorber was added into the separatory funnel, followed by 100 cm³ of distilled water. The funnel was shaken well to extract the compounds to be analyzed into alkaline water phase. The pH of the separated water layer was adjusted to 1 and the analytes were re-extracted with 50 cm³, 25 cm³, and 10 cm³ of toluene. Joint toluene extracts were dried by filtering through anhydrous sodium sulfate and evaporated to ca. 3 cm³ on a rotary vacuum evaporator (Heidolph-Laborota 4003). The residue was transferred quantitatively into a calibrated test tube and the volume was made up to 1 cm³ with a gentle stream of nitrogen.

Isolation of chlorophenols from serum: 100—300 mm³ of serum was placed into a heart-shaped flask. 0.5 cm³ of concentrated HCl was added followed by 50 mm³ of internal standard solution (2,6-DBP, 10.0 μ g cm⁻³). The solution was refluxed for 30 min, cooled,

and the pH adjusted to 2 with 0.5 M-KOH. The sample was diluted with 2 cm³ of water and transferred on the top of a column of 3M Empore high-performance extraction disc cartridges with polystyrene-divinylbenzene copolymer SDB-XC. Prior to use, the column was conditioned with 2.5 cm³ of methanol and 1.5 cm³ of water. Following the application of the sample, the cartridge was dried with a flow of nitrogen. The column was eluted with two 500 mm³ portions of methanol.

Identification and analysis of chlorophenols was carried out on Hewlett—Packard 5890A gas chromatograph with split/splitless injector, HP 7673A autosampler equipped with ⁶³Ni ECD or MSD (Hewlett—Packard GCD 1800A). The SIM mode was employed, on the basis of the base ion DCP and MCP 162. Capillary column: PTETM - 5 (30 m × 0.25 mm, FT 0.25 μm), temperature gradient: from 60°C (4 min), rate 10°C min⁻¹, to final temperature 270°C (3 min), temperature of the ECD 300°C, injector temperature 250°C, carrier gas (nitrogen) flow for cGC-ECD 1.2 cm³ min⁻¹, make-up gas nitrogen 15 cm³ min⁻¹, column pressure 70 kPa. For GCD, helium was used as carrier gas at 1.0 cm³ min⁻¹, column pressure 65 kPa, injector temperature 250°C, splitless technique, temperature of the ion source 280°C. Quantitative evaluation of the chromatograms was performed using HP ChemStation. Quantitation was carried out using the calibration curve and internal standard, from 5 content levels with linear detector response.

RESULTS AND DISCUSSION

Our monitoring studies in the last ten years showed that from among chlorinated phenols, 2,4-DCP occurred at the highest levels both in waters and in total diet [10, 23]. Therefore, 2,4-DCP was chosen for studying simultaneous effect of chlorinated phenols and some natural antioxidants in experiments on guinea pigs.

Published methods for isolation of chlorophenols from liquid matrices by solvent extraction report good recoveries but they are time-consuming and require high volumes of solvents [14—16]. For 2,4-DCP, methods have been published for isolation from human and rat urine, muscle, and liver by extraction with hexane following acid or alkaline hydrolysis of sulfate or glucuronic conjugates. Recoveries in the range of 64 to 78 % and detection limits between 0.02 mg kg⁻¹ and 0.01 mg kg⁻¹ were reported [5]. However, formation of difficult emulsions was often observed. This problem can be avoided by using isolation procedures based on steam distillation or distillation with organic solvent resulting in cleaner extracts. Moreover, these procedures are universal for liquid as well as solid samples. In our previous work we designed an all-glass apparatus for the isolation of pentachlorophenol and isomers of di-, tri-, and tetrachlorophenol from foods, soil, and water by simultaneous extraction with toluene and water vapour [10, 23]. The method is suitable for samples where sample mass is not a limiting factor. For the analysis of 2,4-DCP and its main metabolite 4-MCP in guinea pigs organs (liver, kidney, brain, adipose tissue) we have modified the apparatus originally designed for isolation of PCP, tetrachlorophenol and trichlorophe-

Table 1. Characteristics of the Analytical Method for the Determination of 2,4-DCP in Guinea Pig Tissues and Serum (Based on 10 Analyses)

Matrix	LOQ	Content	Recovery, RSD
	μg g ⁻¹		%
Liver	5 × 10 ⁻⁴	0.01	88.1 ± 5.9
		0.10	92.7 ± 4.3
		1.00	96.5 ± 2.9
		10.00	97.3 ± 4.1
		0.005	81.2 ± 6.2
Kidney, brain	5 × 10 ⁻⁴	0.10	84.7 ± 3.4
		1.00	85.2 ± 3.4
		5.00	87.7 ± 4.9
		0.01	89.5 ± 4.2
		0.10	90.5 ± 2.4
Adipose tissue	5 × 10 ⁻⁴	1.00	91.6 ± 3.8
		2.00	88.3 ± 3.8
		0.01	89.9 ± 4.8
		0.10	95.1 ± 4.7
		0.50	94.1 ± 2.6
Blood serum	5 × 10 ⁻⁴	1.00	91.5 ± 5.7

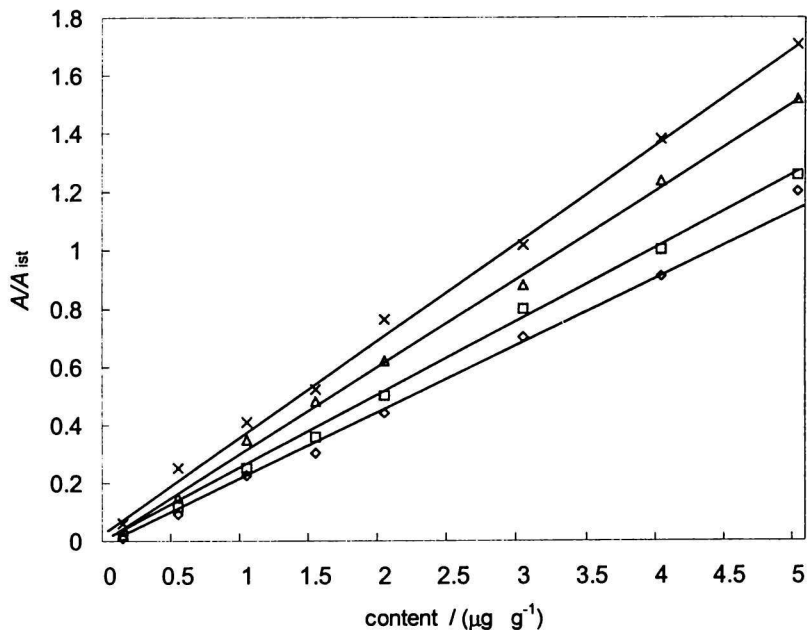


Fig. 2. Calibration curves for the determination of 2,4-DCP in guinea pigs organs. \diamond Liver, \square kidney, Δ brain, \times fat tissue.

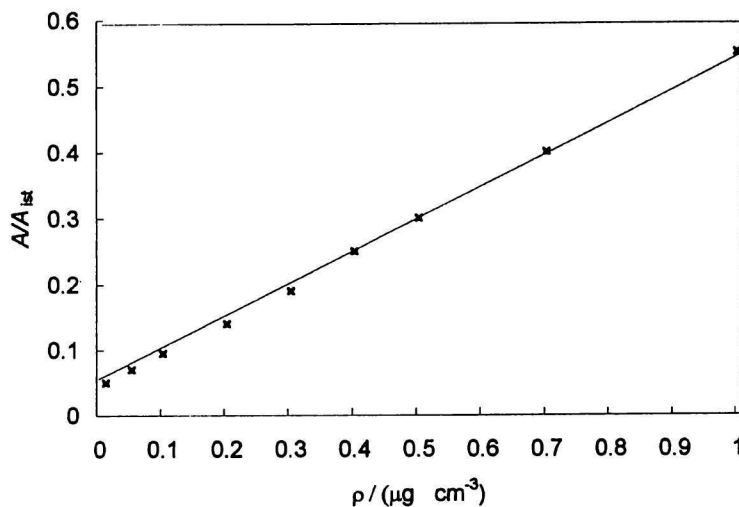


Fig. 3. Calibration curves for the determination of 2,4-DCP in guinea pigs blood serum.

nol isomers from cows milk, fat, and meat [9]. The size of the apparatus has been modified to suit the extraction of very small sample masses with solvent heavier than water (dichloromethane). 2,4-Dichlorophenol recoveries for content levels from 0.005 to 10.0 $\mu\text{g g}^{-1}$ in liver, kidney, brain, and adipose tissue ranged between 81.2 % and 97.3 %.

For isolation of 2,4-DCP and its metabolite 4-MCP from blood serum, a method using Empore high-performance extraction disc cartridges with polystyrene-divinylbenzene copolymer SDB-XC was developed. The extraction disc can tolerate short-term contact with samples having low pH without damaging the copolymer, which results in high recoveries and good reproducibility. The recoveries for contents from 0.01 to 1.0 $\mu\text{g g}^{-1}$ ranged between 89.9 % and 95.1 %. Liquid extraction with toluene resulted in lower recoveries

(82.3 to 88.2 %) for the same concentration range, possibly due to the necessary evaporation of the toluene extracts prior to GC analysis.

The phenols could be analyzed on the capillary PTETM - 5 column in free form, without derivatization.

The recovery, reproducibility, and accuracy of the method have been determined in 10 model experiments with samples from the control animals group (Table 1). Calibration curves were made by adding the standards to the respective matrices. Figs. 2 and 3 show the calibration curves for liver, kidney, brain, adipose tissue, and blood serum. The linear regressions for the respective matrices were as follows: Liver: $y = 0.241146x - 0.03082$, correlation coefficient $r = 0.99763$; kidney: $y = 0.254756x - 0.00579$, $r = 0.99825$; brain: $y = 0.30248x + 0.01119$, $r = 0.99794$;

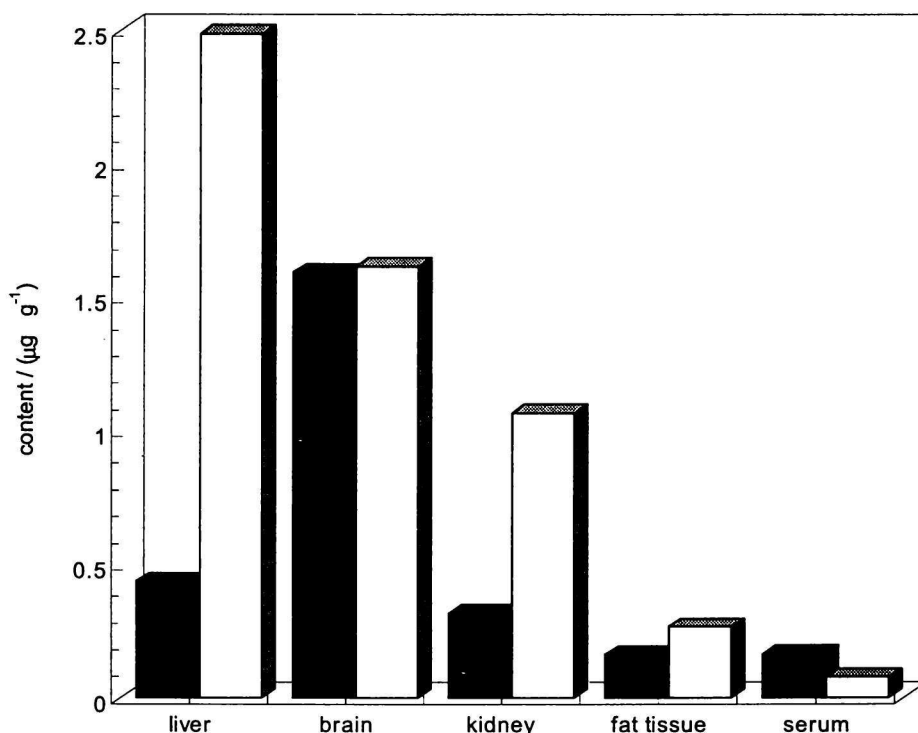


Fig. 4. Average levels of 2,4-DCP in guinea pigs organs and serum at two ascorbic acid (AA) doses. ■ 50 mg/day, □ 2 mg/day.

Table 2. Average Contents of 2,4-DCP and 4-MCP in Guinea Pigs Organs at Two Ascorbic Acid Doses

Tissue type	2 mg day ⁻¹ per animal		50 mg day ⁻¹ per animal	
	2,4-DCP	4-MCP	2,4-DCP	4-MCP
	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹	µg g ⁻¹
Liver	2.486	0.536	0.435	0.110
Kidney	1.065	0.167	0.314	0.038
Brain	1.616	0.710	1.593	0.739
Blood serum	0.076	0.040	0.162	0.083

adipose tissue: $y = 0.32974x + 0.05837$, $r = 0.99745$; blood serum: $y = 0.5059x + 0.04174$, $r = 0.99951$.

The analytical results of the organs of animals fed by 50 mg and 2 mg AA doses are in Fig. 4. In the control groups that did not receive 2,4-DCP the 2,4-DCP levels were between $0.000 \mu\text{g g}^{-1}$ and $0.006 \mu\text{g g}^{-1}$ in the organs and between $0.000 \mu\text{g cm}^{-3}$ and $0.014 \mu\text{g cm}^{-3}$ in the serum.

The results (Fig. 4) document lower accumulation of dichlorophenol in organs of animals fed by higher AA dose. The differences were most pronounced in liver and kidney where the accumulation was lower by factors of 5.7 and 3.4, respectively. No significant differences between the two animal groups were found in brain. In blood serum, the situation was reversed, i.e. there were double concentrations of 2,4-DCP in guinea pigs fed by the higher AA dose, both in average and in median values.

Average contents of 4-MCP as well as those of 2,4-DCP are given in Table 2. The comparison of the levels of the two compounds shows that the relative contents of 4-MCP were the highest in blood serum and in brain, namely 44 to 53 % of the contents of 2,4-DCP. In liver and kidney the 4-MCP levels were from 12 to 25 % of those of 2,4-DCP. No significant differences in 4-MCP levels were found between the two animal groups.

CONCLUSION

The analytical method developed allows determination of 2,4-DCP and 4-MCP in the organs and blood serum of guinea pigs by gas chromatography with ECD and/or MSD. The isolation of the analytes from the organs occurs by steam distillation with dichloromethane following alkaline hydrolysis. From

the blood serum, the compounds were isolated by solid phase extraction on Empore discs and elution with methanol.

The method was used for studying the influence of ascorbic acid antioxidant on pathological effect of 2,4-DCP in guinea pigs. It has been found that saturation of the tissues with high doses of ascorbic acid decreased the accumulation of 2,4-DCP in the tissues and mildened metabolic and pathomorphologic disorders induced by this compound or its transformation products.

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