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A new diquat derivative appropriate for colourimetric measurements of biological materials in the presence of paraquat

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Abstract A new colourimetric method is described for the quantification of diquat using a yellow-coloured derivative produced by heating diquat in alkaline solution at 80°C. The absorption maximum of the yellow derivative is 420 nm and the molar absorption coefficient is 2.76×10^4 (0.15 in 1 μg diquat/ml with 1 cm light path). The absorption at 420 nm shows a linear concentration dependence in the range 0.1–10 μg/ml and fading of the colour is about 5% after 1 h. Under the same conditions, paraquat does not produce any coloured products. The concentration of diquat in the solution containing both diquat and paraquat can be determined by the absorption of diquat derivative at 420 nm without interference from paraquat. By adding sodium dithionite to the solution the concentration of paraquat can be determined by the absorption of paraquat radicals at 600 nm without interference from diquat, because the yellow derivative does not react with dithionite. This yellow diquat derivative can be extracted completely with cyclohexanol by saturating the solution with Na_2SO_4. The absorption maximum in cyclohexanol shifts to 440 nm with the same molar absorbance and the same half-band width as in water. Fading of the colour is less than 5% after 24 h in cyclohexanol. Perchloric acid (3%) and trichloroacetic acid (4.5%) which are often used for deproteinization of tissue homogenates, do not inhibit production of the coloured derivative at pH 13.5 or extraction of the derivative with cyclohexanol. This method is suitable for a quick determination of small amounts of diquat in tissues, since the extraction with cyclohexanol not only concentrates the derivative rapidly but also quite efficiently eliminates the coloured substances in tissue homogenates. The detection limit of diquat is 0.02 μg/ml for blood and 0.05 μg/g for liver when 1 ml or 1 g is used for analysis. In three human cases of fatal intoxication, both paraquat and diquat were quantified using 50 μl of serum. In non-toxic dosing of diquat to rats for 14 days, the diquat level was highest in the spleen followed by the kidneys.

Key words Diquat · Paraquat · Colourimetry · Tissue concentration · Cyclohexanol

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Introduction

The toxicity of the herbicide paraquat (PQ, 1, 1′-dimethyl-4, 4′-dipyridylium) is higher than that of diquat (DQ, 1, 1′-ethylene-2, 2′-dipyridylium) for mammals e.g. the acute oral LD50 of PQ is 30 mg/kg whereas that of DQ is 100 mg/kg in guinea-pigs [1, 2]. To lower the toxicity of herbicides, commercial products containing equimolar amounts of PQ and DQ have been used in Japan since 1986. Although equimolar amounts of PQ and DQ were ingested in cases of poisoning, the molar ratio of DQ/PQ contained in the tissues of the victims varied from 0.08 to 8 indicating differences in the pharmacokinetics of PQ and DQ [3]. It would therefore be desirable to establish a simple method of measuring the concentrations of PQ and DQ in tissues. Because of high aqueous solubility, PQ and DQ are usually determined by liquid chromatography (LC) [4, 5]. The colourimetry of PQ radicals produced with alkaline dithionite [6] is a simple method and still applicable to the quantification of PQ at fatal levels [7]. The optical density (OD) of the PQ radical at 600 nm is 0.0855 for 1 μg/ml [6], however, the alkaline dithionite reaction is not suitable for the colourimetric quantification of DQ because the DQ radical produced is unstable and the absorption is too weak,i.e. the OD at 430 nm is 0.0255 for 1 μg DQ/ml [6]. Therefore, we tried to establish a new colourimetric method for the quantification of DQ, and found that DQ changed to a yellow-coloured derivative when heated at 80 °C in alkaline solution which has higher molar absorptivity as well as higher stability than the DQ radical or the red DQ derivative reported previously [8]. The extraction of the derivative into organic

solvents was also examined since turbid and coloured substances in tissue homogenates often interfere with colourimetric measurements.

Materials and methods

DQ dibromide monohydrate ($C_{12}H_{12}N_2Br_2.H_2O$) was kindly donated by Zeneca K. K. Agrochemicals, Japan and PQ dichloride ($C_{12}H_{14}N_2Cl_2$) was obtained from Wako Chemical, Japan. The standard DQ and PQ solutions were made by measuring the ultraviolet spectra. The molar absorptivity of DQ (ε_{310}) is 19,200 and that of PQ (ε_{256}), 20,600 [6]. All other chemicals used were of analytical grade.

Biological samples

The elimination of impurities and recovery of herbicides from biological materials were studied using blood, serum and urine from healthy humans and tissues of untreated Wistar-strain rats. The applicability of the method was examined using samples from cases of human fatal intoxication and rats after non-toxic dosing. Human sera were obtained from two males and one female who committed suicide with a herbicide containing a 1 : 1 mixture of PQ and DQ. In our previous work the PQ levels had been measured in several organs of rats fed with 180 ppm PQ in the diet although some rats had died before 14 days [9]. The DQ levels, however, could not be measured in any organs of rats fed with 180 ppm DQ in the diet for 14 days. Therefore, to obtain information on the effect of environmental DQ pollution, rat tissue samples were obtained from six Wistar-strain male rats fed with the diet containing 590 ppm DQ for 14 days which is equivalent to 75% of the maximum non-toxic dose level to specific pathogen-free rats for 2 years [2]. The rats were terminated by cardiac puncture under nembutal anaesthesia and the organs were removed after perfusion with cold physiological saline through the portal vein.

Conditions of pH and temperature

The solution containing DQ was preheated in a water bath to reach the desired temperature (30–95 °C), made alkaline with 10 N NaOH (0.2–2 N), maintained for 30 s at the temperature, and then cooled to room temperature. The OD of the solution was measured using a Shimadzu UV2200 spectrophotometer with a pair of quartz micro-cuvettes (volume 0.15 ml) of 1 cm path length.

Inhibitors

The inhibitors examined were perchloric acid (PCA), trichloroacetic acid (TCA), sodium dithionite, cysteine, ascorbic acid and formalin. The solution containing both DQ and one of the inhibitors was preheated at 80 °C and 10 N NaOH solution was added to pH 13.5. The solution was maintained at 80 °C for 30 s before measuring the OD. The final concentration of the inhibitor ranged from 10^{-4} to 10^{-1} g/ml.

Extractability into organic solvents

The yellow DQ derivative produced in 1 ml of NaOH solution at pH 13.5 was added to one of the organic solvents (1 ml) 1-butanol, 2-methyl-1-propanol, cyclohexanol, 1-pentanol, 3-methyl-1-butanol, 1-hexanol, 1-octanol, 2-butanol, ethyl acetate, diethyl ether, dichloromethane, chloroform, pentane, hexane, benzene and toluene. Solutions were vortexed for 1 min and centrifuged at 13,000 g for 1 min. The OD of the organic layer was measured at 440 nm and that of the aqueous layer at 420 nm. When the organic layer was too turbid for OD measurement a small amount of solid Na_2SO_4 was added to the organic layer to reduce the water content or a small amount of 1-butanol was added to the organic layer to increase the solubility of water.

Effects of salts on the extraction

The yellow DQ derivative produced in 5 ml of NaOH solution at pH 13.5 was added to one of the alcohol solvents (0.4 ml) and one of the following salts; Na_2SO_4, Na_2CO_3, K_2CO_3, K_3PO_4, NaCl or KCl. The amount of the salts added was 0.3 g/ml aqueous solution and solutions were vortexed for 1 min and centrifuged at 13,000 g for 1 min.

Elimination of coloured impurities

Tissue samples (1 g) were taken from the lungs, spleen, kidney or liver, or 1 ml of blood was homogenized with 3 ml of water by a polytron homogenizer (Kinematica, Luzern, Switzerland) in a centrifuge tube. To obtain 3% PCA homogenate, a suitable amount of 70% PCA solution was added drop-wise to the stirred homogenate to avoid large clots, the homogenate was centrifuged at 13,000 g for 2 min and the supernatant was transferred to another centrifuge tube. The precipitate was washed once with 0.5 ml of 3% PCA solution and the resulting supernatant was added to the first supernatant. The pH of the supernatant was adjusted to 13.5 by adding 0.6 ml of 10 N NaOH solution, and the total volume was adjusted to 5 ml by adding water. For 1 ml of serum, a suitable amount of 70% PCA solution was added to obtain 3% PCA serum. Then the homogenate was centrifuged at 13,000 g for 2 min, and the supernatant was transferred to another tube. The precipitate was washed once and the pH of the combined supernatant was adjusted to 13.5 with 10 N NaOH. The final volume of the supernatant of serum was 1 ml. The pH of urine samples was adjusted to 13.5 with 10 N NaOH. The solutions from the biological materials were maintained at 80 °C for 30 s and the OD was measured at 440 nm to compare the amounts of coloured impurities in the supernatants. To these solutions, Na_2SO_4 (0.3 g/ml) and 0.4 ml of one of the alcohols 1-butanol, 2-methyl-1-propanol, cyclohexanol, 1-pentanol or 3-methyl-1-butanol were added, mixed with a vortexer for 1 min and centrifuged at 13,000 g for 1 min. The OD at 440 nm of the alcohol layer was measured to compare the capability of the alcohols to eliminate impurities.

Recovery of the herbicides in spiked materials

Lung, spleen, kidney or liver samples (1 g) or 1 ml of blood, serum or urine were spiked with 2 μg each of DQ and PQ, and homogenized as described. The DQ derivative was produced by adding 0.6 ml of 10 N NaOH solution to the deproteinized supernatant preheated at 80 °C and the solution was maintained at 80 °C for 30 s. The volume of the solution and the absorption of DQ derivative at 420 nm were measured to calculate the amount of DQ, sodium dithionite was added to a final concentration of 0.1% and the absorption of PQ radicals at 600 nm was measured to calculate the amount of PQ. To the solution, 0.4 ml of cyclohexanol and Na_2SO_4 (0.3 g/ml aqueous solution) were added, vortexed for 1 min and centrifuged at 13,000 g for 1 min. The OD of the cyclohexanol layer was measured at 440 nm to calculate the amount of DQ.

Results and discussion

Figures 1 and 2 show the fraction of the yellow DQ derivative produced as a function of the concentration of NaOH and that of temperature, respectively. The suitable range of NaOH concentration was found to be from 0.4 to 0.6 N, corresponding to pH 13.3–3.7 and that of temperature was from 70 to 95 °C indicating the relative ratio of the yellow derivative produced under the indicated condi-

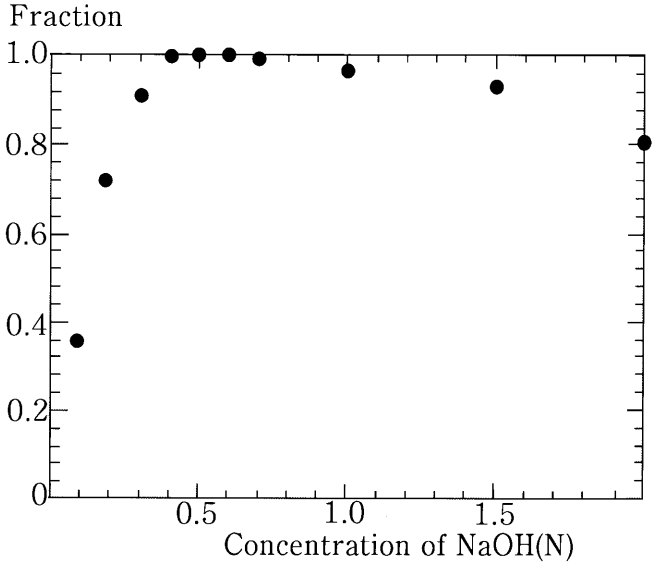

Fig. 1 Effect of NaOH concentration on the diquat derivative production. Diquat was added to each NaOH solution preheated at 80 °C and the solution was maintained at 80 °C for 30 s

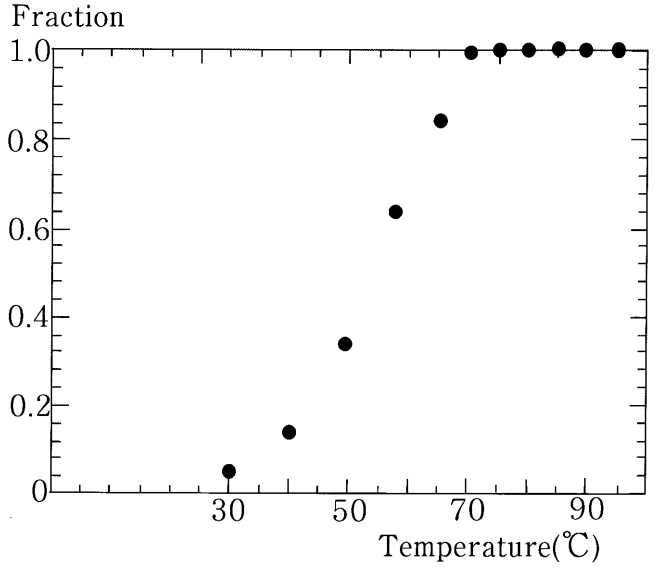

Fig. 2 Effect of temperature on the diquat derivative production. Diquat solution was preheated at the temperature indicated, and was maintained at the temperature for 30 s after adjusting the NaOH concentration to 0.5 N

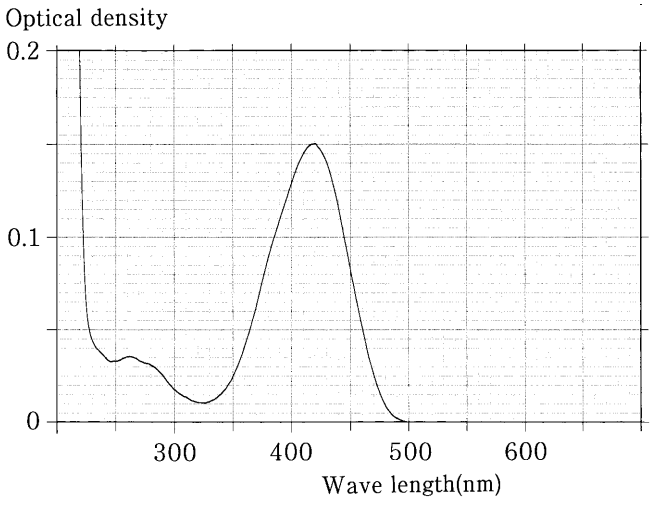

Fig. 3 Absorption spectrum of the diquat derivative produced from 1 μg diquat/ml in 0.5 N NaOH solution

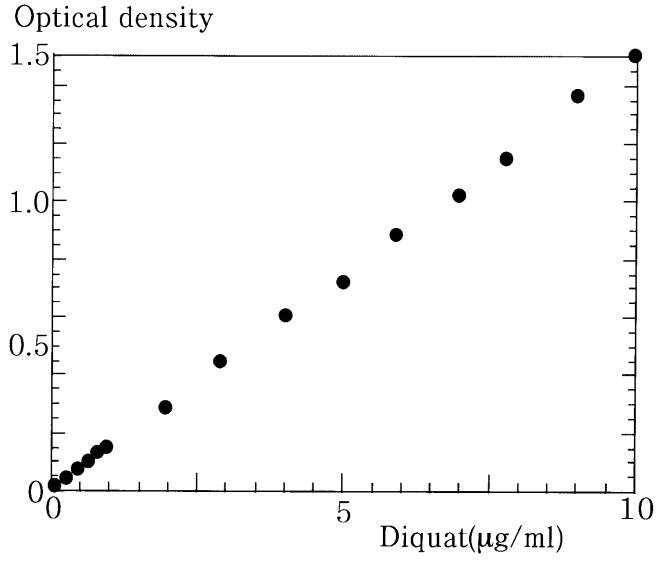

Fig. 4 Calibration curve relating absorbance at 420 nm to diquat concentration

tion to that produced under the most suitable condition, i.e. 80 °C in 0.5 N NaOH solution.

The absorption spectrum of the yellow DQ derivative produced at 80 °C in 0.5 N NaOH solution is shown in Fig. 3. The peak with the OD of 0.150 at 420 nm was observed for 1 μg DQ/ml solution and no absorption was detected at 600 nm which is the wavelength of the absorption peak of PQ radical.

The linearity of the absorption of the derivative was established for the concentration range from 0.1 to 10 μg/ml as shown in Fig. 4. The yellow colour is clearly visible even in 0.5 μg/ml solution and Fig. 5 indicates the relationship between the concentration of inhibitor and the fraction of the yellow derivative produced in the inhibitor solution at pH 13.5. A solution of either formalin, ascorbic acid or cysteine below 0.01% or sodium dithionite solution below 0.1% did not inhibit derivative production. Although formalin solutions above 0.01% decomposed the yellow derivative produced, sodium dithionite, ascorbic acid or cysteine solutions at concentrations below 5% did not. TCA or PCA solutions below 10% neither inhibited the production nor decomposed the derivative.

Organic solvents such as ethyl acetate, diethyl ether, dichloromethane, chloroform, pentane, hexane, benzene and toluene could not extract the yellow derivative, whereas alcohols could, as shown in Table 1. Among the alcohols, 1-butanol extracted the derivative most efficiently. The effect of salts on the extraction was examined

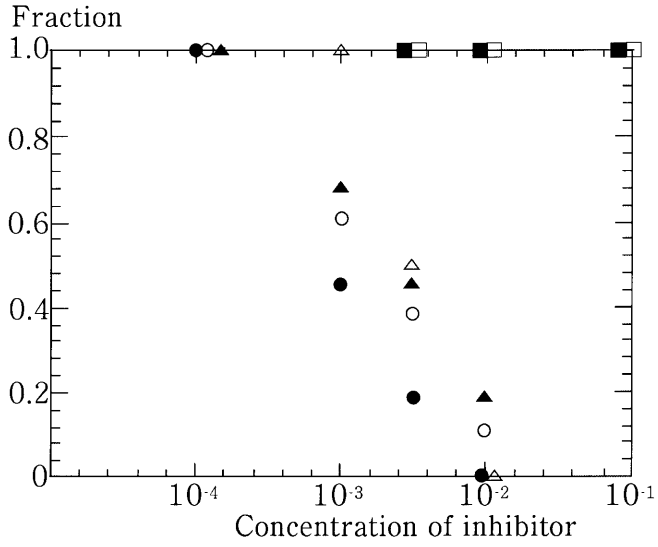

Fig. 5 Effect of inhibitors on the diquat derivative production. ● formalin, ○ ascorbic acid, ▲ cysteine, △ sodium dithionite, ■ trichloroacetic acid, □ perchloric acid

Table 1 Percentages of the yellow diquat derivative extracted with alcohol layer in alcohol-water mixtures (*0.3 g Na_2SO_4/ml water)

	Without Na_2SO_4 Alcohol : water (1 : 1)	With Na_2SO_4* Alcohol : water (0.4 : 5)
1-Butanol	73	> 98
2-Methyl-1-propanol	56	> 98
Cyclohexanol	52	> 98
1-Pentanol	38	> 98
3-Methyl-1-butanol	33	> 98
1-Hexanol	25	90
1-Octanol	10	40
2-Butanol	10	40

using Na_2SO_4, Na_2CO_3, K_2CO_3, K_3PO_4, NaCl and KCl (0.3 g/ml aqueous solution) and complete extraction was achieved by the combination of either 1-butanol, 2-methyl-1-propanol, cyclohexanol, 1-pentanol or 3-methyl-1-butanol and either Na_2SO_4, Na_2CO_3, K_2CO_3 or K_3PO_4. The extraction was about 90% by the combination of 1-butanol and either NaCl or KCl or by the combination 1-hexanol and Na_2SO_4 but was only 40% by the combination of either 1-octanol or 2-butanol and Na_2SO_4 (Table 1). Salts not only enhanced the extraction of the yellow derivative into the alcohol layer but also facilitated the separation of a small amount of alcohol layer from the aqueous layer. When the volume ratio of cyclohexanol to aqueous solution was 0.4 ml : 5 ml, respectively, as in the present experiment, the concentration of the yellow DQ derivative in cyclohexanol was 12.5 times that in aqueous solution.

The absorption spectrum of the derivative in alcohols is almost the same as that in aqueous solution. For example, the molar absorptivity and the half-band width in cyclohexanol are the same as those in water although the peak is shifted to 440 nm in cyclohexanol. The yellow derivative is quite stable in alcohols i.e., fading of the colour is less than 5% after 24 h in cycohexanol.

Tissues contain coloured substances whereby the OD at 440 nm was 0.6 for kidney, 0.5 for liver, 0.22 for spleen 0.13 for heart, 0.05 for lung tissue and 0.05 for blood, for 5 ml of deproteinized supernatant with pH 13.5 derived from 1 g or 1 ml of each biological material. The OD at 440 nm was 0.1 for 1 ml of deproteinized supernatant with pH 13.5 derived from l ml of serum and about 0.2 for urine with pH 13.5. Therefore some supernatants cannot be used for the quantification of the DQ derivative. Since complete extraction of the DQ derivative was achieved by five alcohols such as 1-butanol, 2-methyl-1-propanol, cyclohexanol, 1-pentanol and 3-methyl-1-butanol as shown in Table 1, the ability to eliminate coloured impurities in biological materials was compared. The deproteinized supernatant with pH 13.5 from liver was used for the comparison of alcohols, because liver contained the highest amount of coloured impurities soluble in alcohols among the tissues and body fluids. The OD at 440 nm of 0.4 ml of the alcohol layer mixed with 5 ml of liver supernatant and Na_2SO_4 was 0.08 for 1-butanol, 0.05 for 2-methyl-1-propanol, 0.04 for 1-pentanol, 0.04 for 3-methyl-1-butanol and 0.02 for cyclohexanol, indicating that cyclohexanol is the most suitable alcohol for DQ quantification in biological materials. When cycohexanol was used the impurity elimination rate was about 300 times greater since the OD of 5 ml of the supernatant is 0.5 and that of 0.4 ml of the cycohexanol layer is 0.02. The OD at 440 nm of 0.4 ml of the cyclohexanol layer due to impurities extracted from 1 g or 1 ml of biological materials was 0.02 for liver, 0.015 for spleen, 0.01 for kidney, heart or lung, and less than 0.002 for blood, serum or urine.

The instrumental limit of detection (signal-to-noise ratio=3) at 440 nm corresponds to 0.02 μg DQ per 1 ml biological fluid or 1 g tissue. This limit is applicable to blood, serum and urine, because cyclohexanol does not extract impurities from these materials above the noise level, but cyclohexanol extracts impurities from tissues, especially from liver. According to the OD of impurities at 440 nm, the limit of detection was 0.05 μg/g for liver, 0.04 μg/g for kidney and 0.03 μg/g for spleen, heart and lung. The recovery of DQ in cyclohexanol was more than 85% and that of PQ in supernatant more than 90%, for all materials spiked with 2 μg each of DQ and PQ.

Although the yellow DQ derivative was not decomposed by sodium dithionite solution below 5%, PQ radical production by sodium dithionite was partly inhibited by salts such as Na_2SO_4, Na_2CO_3, K_2CO_3, K_3PO_4, NaCl or KCl at concentrations of 0.3 g/ml. Therefore, the most efficient colourimetric determination of the mixture of PQ and DQ in biological materials is as follows:

Step 1. Maintain the deproteinized supernatant at 80 °C and pH 13.5 for 30 s and measure the OD of the yellow DQ derivative at 420 nm

Step 2. Add 1 mg sodium dithionite/ml to the solution and measure the OD of the blue PQ radical at 600 nm

Step 3. Add 0.4 ml of cyclohexanol and Na_2SO_4(0.3 g/ml water) to the solution, mix for 1 min and centrifuge at 13,000 g for 1 min. Then measure the OD of the yellow DQ derivative at 440 nm in cyclohexanol

The herbicide levels were measured in three cases of fatal human intoxication by this method using 50 µl serum which was diluted to 1 ml and deproteinized. The DQ and PQ levels were measured according to steps 1 and 2, the DQ levels were found to be 84, 82, and 33 µg/ml and the PQ levels 118, 104 and 39 µg/ml, respectively.

Although 180 ppm PQ in the diet was toxic to rats [9], 180 ppm DQ was found to be non-toxic, and the DQ levels could not be measured in any organ. Therefore, tissue DQ levels were measured for the six rats fed with 590 ppm DQ for 14 days according to steps 1 and 3 and the levels were 2.3 ± 0.7, 0.38 ± 0.07 and 0.05 ± 0.02 µg/g for spleen, kidney and lung, respectively, and 0.04 ± 0.02 µg/ml for serum, but for liver and heart the levels were below the detection limit.

A solid-phase extraction method reported by Ibanez et al. [4] and Taguchi et al. [5] could concentrate a dilute DQ solution to nearly 1000-fold. The LC-ultraviolet detection method and LC-mass spectrometry are quite suitable methods for the quantification of DQ in environmentally polluted crops and water. The detection limit of 0.1 µg/l is quite low but a large amount of sample (250–1000 ml) is required [4, 5] and the mass detection limit is around 25 ng. Although liquid-phase extraction such as our present method is simple and quick, the solution could only be concentrated to 10-fold its original volume. Our method is suitable for the quantification of DQ in small tissues samples of around 1 ml or 1 g, and the mass detection limit is 20 ng for blood or 50 ng for liver, which is of comparable order to the mass detection limit reported by these authors.

The yellow DQ derivative is produced from DQ irreversibly in alkaline solution at 80 °C and does not return to DQ on acidification. Therefore, new absorption peaks which do not correspond to those of DQ appear at 270 nm and 350 nm on acidification. Although the yellow derivative can be extracted with alcohols and is much more stable in alcohols (less than 5% decay after 24 h in 1-butanol or in cyclohexanol) than in aqueous solution, it can not be produced in 1-butanol saturated with 10 N NaOH solution at 80 °C.

The DQ concentration in body fluids and organs was found to be relatively lower than those of PQ except in bile and kidney when the same amounts of PQ and DQ were ingested [3]. The difference in distributions of PQ and DQ in the body may be caused by differences in absorption, excretion and metabolism. The metabolic turnover rate of DQ is higher than that of PQ in liver homogenates [10]. The present yellow derivative does not correspond to either of the two metabolites reported previously [11], because the absorption peak of both metabolites is 357 nm and both metabolites are quite soluble in chloroform, whereas this derivative absorbs light at 420 nm and is insoluble in chloroform. DQ is photodegraded more easily than PQ [12, 13] but the present yellow derivative is none of the photodegraded products reported previously[13] because they only absorb light at wavelengths shorter than 270 nm [12]. Previously we reported a red DQ derivative which was produced by moderate reduction in alkaline pH [8]. The present yellow derivative has a higher molar absorptivity (ε_{420} = 27 600) than DQ itself (ε_{310} = 19 200) [6], its radical (ε_{430} = 4140) [6], its metabolites (ε_{357} = 14 900) [11] and its red derivative (ε_{495} = 20 700) [8] reported before. We are now investigating the structure of the derivative. The results of LC-mass spectrometry at present, however, show only fragments which are too small to allow an estimation of the molecular structure. ESR spectroscopy indicates that this derivative is not a radical. Other methods are needed to clarify its structure.

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