Selective Kinetic Determination of Paraquat Using Long-Wavelength Fluorescence Detection

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The reaction between paraquat, ascorbic acid, and Cresyl Violet in alkaline medium and in the presence of sodium dodecyl sulfate has been applied for the first time to the development of a kinetic−fluorometric method for the determination of paraquat. The reaction rate of this system is measured by using the stopped-flow mixing technique, which makes the method applicable to automatic routine analysis. Analytical data are obtained in ∼30 s. The calibration graph is linear over the range 6−500 ng mL^{-1}, and the detection limit is 1.8 ng mL^{-1}. The relative standard deviation is <3%. The use of dynamic measurements at long wavelength favors the high selectivity of the method. Diquat behaves in this system similarly to paraquat, but its interferent effect is easily avoided by using cysteine. The proposed method has been applied to the determination of paraquat in tap water, milk, and white wine samples with recoveries of 89−104%.

Keywords: *Paraquat; Cresyl Violet; kinetic method; stopped-flow; liquid food samples*

INTRODUCTION

The wide use of paraquat (1,1′-dimethyl-4,4′-dipyridinium ion) in agriculture and horticulture and its highly toxic effect on humans justify the numerous methods for the control of this herbicide in environmental, food, and clinical samples that have been reported. Chromatography (Worobey, 1993; Kambhampati et al., 1994) and electrophoresis (Wigfield et al., 1993; Kaniansky et al., 1994) have been mainly applied to the determination of paraquat together with other herbicides such as diquat, although some chromatographic methods have been described for the individual determination of paraquat (Ahmad, 1983; Corasaniti and Nistico, 1993; Croes et al., 1993). Some photometric methods have been also reported. The majority of them are based on the reduction of paraquat in alkaline medium to a blue radical cation, which absorbs at 600 nm. Ascorbic acid (Shivhare and Gupta, 1991; Jain et al., 1993), sodium dithionite (Kuo, 1986), and sodium borohydride (Rai et al., 1997) have been used as reductant agents. Thus, the method based on the use of sodium dithionite was adopted by the AOAC for the determination of paraquat in pesticide formulations (AOAC, 1990). The lowest quantification limit reported by using ascorbic acid or sodium dithionite was 100 ng mL^{-1}, and that by using sodium borohydride was 50 ng mL^{-1}. However, the development of an integrated continuous retention−reaction−detection flow approach (Agudo et al., 1993) with the dithionite method has allowed the quantification limit to be decreased to 0.44 ng mL^{-1}, although the sampling frecuency was very low (0.9 h^{-1}) because the analysis required large sample volumes (250 mL). Another photometric method involves the reaction of paraquat with tetraiodobismutate (Yañez-Sedeño and Polo-Diez, 1986), but the quantification limit obtained is higher than those mentioned above.

Diquat (1,1′-ethylene-2,2′-dipyridinium ion), which is structurally related to paraquat and also forms a stable colored radical, is one of the most serious interferences in the photometric methods for paraquat. Treatment with sodium hydroxide has been recommended for the removal of diquat (Yañez-Sedeño and Polo-Diez, 1986), but the maximum tolerable diquat/paraquat ratio is 1.9 and the treatment takes 5−6 h. Several enzyme immunoassay methods have been reported for paraquat determination (Niewola et al., 1985, 1986; Van-Emon et al., 1986; Selisker et al., 1995), in which the interference of diquat is avoided by using very selective monoclonal antibodies.

Although fluorometry allows very sensitive methods to be obtained, this technique has been rarely applied to the determination of paraquat. A fluorescence polarization immunoassay (Colbert and Coxon, 1988) was described, but no data about detection and quantification limit were given. This paper reports a fast kinetic−fluorimetric method based on the increased reductant effect of the radical cation of paraquat, obtained by reaction of paraquat with ascorbic acid, on the redox reaction between Cresyl Violet and ascorbic acid, in the presence of the surfactant sodium dodecyl sulfate (SDS). Cresyl Violet was chosen as reagent because its fluorescence emission maximum appears at longer wavelength than those of conventional fluorophores, avoiding or minimizing the background interferences from the sample matrix and obtaining the spectral discrimination of the analytical signal. The system has been studied by using a stopped-flow mixing technique, which allows high initial rates to be measured and facilitates automation through automatic mixing of sample and reagent solutions, so that the manipulation is minimal, and measurements are performed shortly after mixing. The proposed method has been applied to the determination of paraquat in liquid food samples, namely, tap water, milk, and white wine.

As the aim of this study has been the development of a very selective method for the determination of

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paraquat, different assays were carried out to avoid the interference of diquat, which causes an effect on the system similar to that of paraquat. Of these assays, the formation of a charge-transfer complex between cysteine and diquat (Pérez-Ruiz et al., 1994) gave the best results, which allows the tolerated level of this compound to be notably increased in relation to those reported in the methods mentioned above.

MATERIALS AND METHODS

Instrumentation. An SLM-Aminco (Urbana, IL) Model 8100 photon-counting spectrofluorometer, equipped with a 450 W xenon arc source and an R928 photomultiplier tube, was used. The instrument was furnished with an SLM-Aminco Milliflow stopped-flow module, which was fitted with an observation cell of 0.2 cm path length and controlled by the associated electronics, a computer and a pneumatic syringe drive system. The solutions in the stopped-flow module were kept at a constant temperature of 40 °C by circulating water from a thermostated tank.

Chemicals. All chemicals used were of analytical reagent grade. Milli-Q water was used throughout. An aqueous stock solution (100 μg mL^{-1}) of paraquat was prepared from 1,1′-dimethyl-4,4′-bipyridylium dichloride (Riedel). Working solutions were prepared daily by appropriate dilution. Aqueous solutions of Cresyl Violet acetate (Sigma) (9.3×10^{-5} M), ascorbic acid (Merck) (2.8×10^{-2} M), SDS (Aldrich) (10^{-2} M), and sodium hydroxide (Merck) (2 M) were also prepared.

Analytical Method. One of the two 2-mL drive syringes of the stopped-flow module was filled with a previous solution containing paraquat, at a final concentration between 6 and 500 ng mL^{-1}, sodium hydroxide (0.8 M), and SDS (10^{-3} M). The other syringe was filled with a solution containing Cresyl Violet (1.4×10^{-5} M), ascorbic acid (2.8×10^{-3} M), and SDS (10^{-3} M). In each run, 40 μL of each solution was mixed at a flow rate of 20 mL s^{-1} in the mixing chamber. The variation of the fluorescence intensity with time throughout the reaction was monitored at $\lambda_{ex} = 467$ nm and $\lambda_{em} = 620$ nm for ~30 s, and the reaction rate was determined in 5 s, once the induction period was finished. The data were processed by the computer, furnished with a linear regression program for application of the reaction rate method. All measurements were carried out at 40 °C. Each standard or sample was assayed in triplicate. A linear calibration graph was obtained by plotting the reaction rate difference obtained in the absence and presence of paraquat versus the paraquat concentration.

Determination of Paraquat in Liquid Food Samples. Three samples (tap water, milk, and white wine) were spiked with appropriate amounts of paraquat. A volume (0.8 mL) of each sample was treated with 0.4 mL of 5% EDTA to avoid the interference of metal ions. This was the only pretreatment required for the analysis of the tap water sample. The milk sample was also treated with 0.4 mL of 1% trichloroacetic for deproteinization and, after centrifugation at 1850*g* for 10 min, the supernatant was analyzed as described above. Finally, the determination of paraquat in the white wine sample required that the calibration graph was constructed by preparing each paraquat standard in the presence of white wine (40%) and EDTA (1%), to compensate the light increase in the reaction rate of the system caused by the sample matrix.

RESULTS AND DISCUSSION

Study of the Chemical System. With the purpose of developing a selective fluorometric method for paraquat determination, its potential interaction with several long-wavelength fluorophores, namely, oxazines such as Cresyl Violet and Nile Blue, and thiazines such as Azure A, Azure B, and Toluidine Blue, was assayed. These compounds were chosen because, as known, they are less prone to spectral interferences from the sample matrix than conventional fluorophores and the probability of nonradiative quenching processes is also less, as they have a shorter fluorescence lifetime (Patonay and Antoine, 1991).

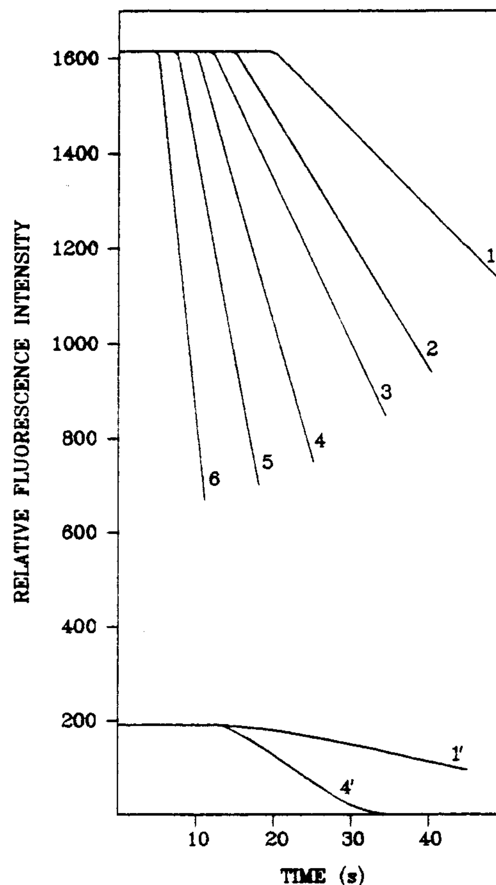

Figure 1. Kinetic behavior of the Cresyl Violet–ascorbic acid system in the presence (curves 1–6) or absence (curves 1′ and 4′) of SDS and in the presence (curves 2–6 and 4′) or absence (curves 1, 1′) of paraquat. [Cresyl Violet] = 1.4×10^{-5} M; [ascorbic acid] = 2.8×10^{-3} M; [SDS] = 10^{-3} M; [NaOH] = 0.8 M; [paraquat] (ng mL^{-1}) = (curves 1 and 1′) 0, (curve 2) 25, (curve 3) 50, (curves 4 and 4′) 125, (curve 5) 250, and (curve 6) 500.

A systematic study at different pH values was carried out in the absence or presence of cationic, anionic, or nonionic surfactants, which could favor the potential interaction of paraquat with these fluorophores, but the different assays did not give any positive result. However, taking into account the fact that paraquat reacts with reductant agents such as ascorbic acid (Shivhare and Gupta, 1991; Jain et al., 1993) or sodium dithionite (Fell et al., 1981; Kuo, 1986) in alkaline medium, giving rise to a radical cation which is more reactive than paraquat, the behavior of the different long-wavelength fluorophores in this alkaline system was also studied. Paraquat did not cause any change in the fluorescent behavior of the different thiazines assayed but notably increased the rate of reduction of Cresyl Violet and Nile Blue by ascorbic acid, its effect on the Cresyl Violet being more pronounced.

A previous study of the fluorescent behavior of Cresyl Violet in alkaline medium showed that its fluorescence ($\lambda_{ex} = 470$, $\lambda_{em} = 620$ nm) is very low and does not change in the presence of a cationic (cetyltrimethylammonium bromide) or nonionic (Triton X-100) surfactant, but increases ~8 times when an anionic surfactant such as SDS is present in the solution. Figure 1 shows this positive effect and, also, the kinetic behavior of Cresyl

Violet in the presence of ascorbic acid alone (curves 1 and 1′) and together with paraquat (curves 2−6 and 4′). As can be seen, the reaction of this dye with ascorbic acid alone in alkaline medium shows an induction period of ~20 s, after which time, the fluorescence decreases slowly, although the decrease is faster in the presence of SDS. However, the induction period decreases and the reaction rate notably increases in the presence of paraquat, and this effect is more intense in the micellar medium and function of the paraquat concentration, as Figure 1 shows. This behavior can be ascribed to the fact that the radical cation formed on the reduction of paraquat is a strong reductant and contributes together with the ascorbic acid to the Cresyl Violet reduction, decreasing rapidly its fluorescence intensity. Although the induction period also decreases when the paraquat concentration increases, the use of the reaction rate as an analytical parameter is more suitable for quantification purposes. Sodium dithionite was assayed instead of ascorbic acid but, in this instance, the reduction of Cresyl Violet was very fast, so that the induction period disappeared and paraquat did not cause any distinctive effect on the system. The fast reaction rate of this system is easily measured by using stopped-flow mixing technique.

The study of the distribution of the reactants between the two syringes of the stopped-flow module showed that the best results are obtained when Cresyl Violet is placed together with ascorbic acid in one syringe, paraquat together with sodium hydroxide in the other, and SDS in both. This distribution avoids the instability of ascorbic acid and Cresyl Violet in alkaline solution and the previous reduction of Cresyl Violet by ascorbic acid, which is favored in the basic medium.

Optimization of Variables. The system was optimized by altering each variable in turn while all others remained constant. All reported concentrations are initial concentrations in the syringes (twice the actual concentrations in the reaction mixture at time zero after mixing). Each kinetic result was the average of three measurements.

The concentration of ascorbic acid in the system is a key variable as it reduces to both paraquat and Cresyl Violet. Figure 2A shows the effect of this variable on the kinetic behavior on the system, in the absence or presence of paraquat. As can be seen, the rate of reduction of Cresyl Violet is very low and remains constant from a 2.4×10^{-3} M ascorbic acid concentration. However, the reaction rate notably increases in the presence of paraquat, the highest values being obtained in the range 2.4×10^{-3}−3.6×10^{-3} M and decreasing at higher values of this variable. The development of the reaction requires a concentrated alkaline medium. Thus, the reaction rate increased when the sodium hydroxide concentration was increased to 0.7 M, remaining constant up to, at least, 1 M. Figure 2B shows that the reaction rate was maximum and practically independent of the Cresyl Violet concentration from 10^{-5} M to, at least, 3×10^{-5} M. The blank signal, obtained in the absence of paraquat, showed only a slight increase in the Cresyl Violet concentration interval assayed.

In relation to the SDS concentration, the best kinetic results were obtained with the surfactant placed in both syringes. Thus, the study of this variable was carried out by changing simultaneously its concentration in both syringes. The reaction rate of the system increased up to a 10^{-3} M SDS concentration, remained constant

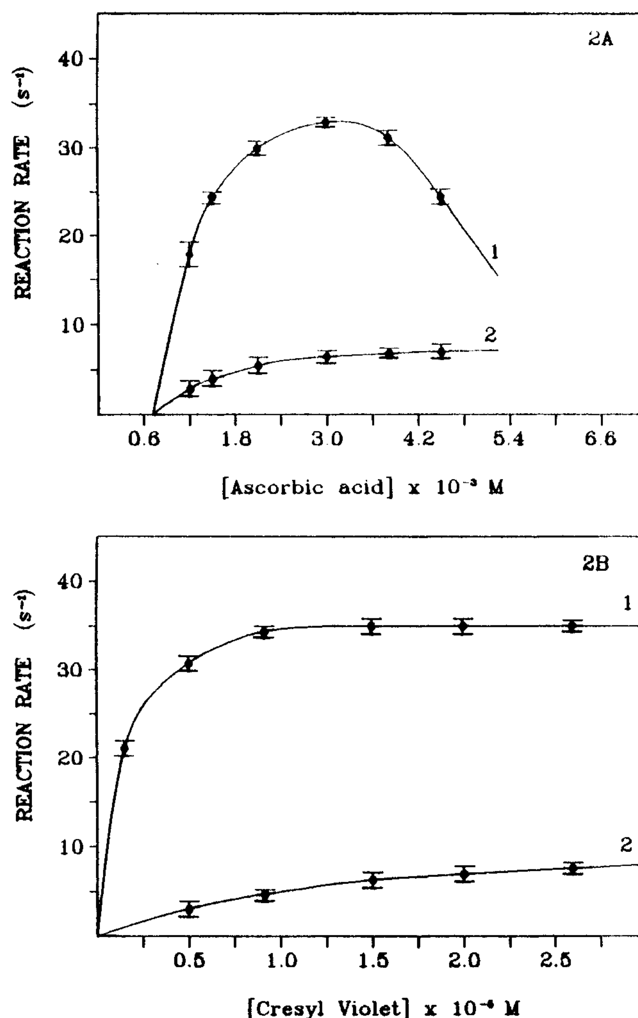

Figure 2. Effect of ascorbic acid (A) and Cresyl Violet (B) concentrations in the presence (curve 1) or absence (curve 2) of paraquat (50 ng mL^{-1}). [SDS] = 10^{-3} M; [NaOH] = 0.8 M; [ascorbic acid] (in B) = 2.8×10^{-3} M; [Cresyl Violet] (in A) = 1.4×10^{-5} M.

up to 1.5×10^{-3} M, and slightly decreased at higher concentrations. Although the critical micelle concentration (cmc) reported (Love et al., 1984) for SDS in pure aqueous solution is 8.1×10^{-3} M, it was measured in this system with a stalagmometer and the value found was 1.2×10^{-3} M, which is within the optimum SDS range obtained for the reaction rate. The decrease of the cmc is logical when one takes into account the strong alkaline medium used (0.8 M). These results show that the micelles perform two functions in the system: first, they shelter the Cresyl Violet molecules from nonradiative processes, increasing the fluorescence intensity of the dye, as described above, and, second, their negative charge favors the reaction between Cresyl Violet and the radical cation formed by reduction of paraquat. Three other anionic surfactants assayed (Tergitol 7, Aerosol OT, and sodium dodecylbenzenesulfonate) provided poorer results than SDS. Finally, the study of the effect of the temperature showed that the reaction rate increases slightly when this variable increased from 25 to 40 °C. Thus, a temperature of 40 °C was chosen.

Analytical Features. The kinetic curves obtained under the optimum conditions for different paraquat concentrations were processed by the reaction rate method. The calibration graph, obtained by using 11

Table 1. Recovery of Paraquat Added in Food Samples

sample	added (ng mL^{-1})	found[a] (ng mL^{-1})	recovery (%)
milk	25	24 ± 5	96
	50	48 ± 3	96
	100	101 ± 7	101
tap water	25	26 ± 1	104
	50	52 ± 1	104
	100	97 ± 3	97
white wine	25	24 ± 1	96
	50	48 ± 2	96
	100	89 ± 2	89

[a] Average of three determinations.

paraquat standards, was linear over the range 6−500 ng mL^{-1}. The Pearson's correlation coefficient (r) was 0.998. The detection limit, as defined by IUPAC (Long and Winefordner, 1983), was 1.8 ng mL^{-1}. The precision of the method was assessed at two concentrations of paraquat, 7 and 150 ng mL^{-1}. The relative standard deviations ($n = 11$) were 1.7 and 2.6%, respectively.

The study of the selectivity of the method showed that other pesticides and related chemicals such as carbaryl, linuron, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, atrazine, simazine, and naptalam are tolerated in a 100-fold excess relative to the analyte. The potential interference of metal ions that form hydroxides in the basic medium used is easily prevented by the addition to each sample of 0.4 mL of 1% EDTA solution prior to the addition of the sodium hydroxide solution. The most serious interference of the method is caused by diquat, which behaves similarly to paraquat in the system. Although the treatment with sodium hydroxide of the samples containing both paraquat and diquat has been recommended for the removal of diquat (Ganesan et al., 1979; Yañez-Sedeño and Polo-Diez, 1986), the assays carried out in the fluorescent system did not give satisfactory results. However, the formation of a charge-transfer complex between diquat and cysteine (Pérez-Ruiz et al., 1994) allows the interference of diquat to be easily eliminated. Thus, the sample containing paraquat and diquat was treated with cysteine (2×10^{-3} M) in the presence of sodium hydroxide (0.8 M) and SDS (10^{-3} M) at 40 °C for 5 min. Although higher cysteine concentrations could alter the behavior of paraquat in the system, the use of this cysteine concentration did not cause any effect on the analytical signal. The procedure allows diquat to be tolerated in a 100-fold excess relative to the analyte, which is higher than the tolerated levels described for diquat in the photometric methods mentioned above and avoids also the physical separation of this interferent species by using a technique such as chromatography or electrophoresis, which is time-consuming.

Applications. To check the analytical usefulness of the proposed kinetic method for the determination of paraquat, three liquid food samples (tap water, milk, and white wine) were spiked with different amounts of paraquat (Table 1). As described above, all of the samples were treated with EDTA to remove the interference by metal ions. Deproteinization with trichloroacetic acid was required for the analysis of the milk sample to avoid the reaction of SDS with proteins, as this surfactant is a protein denaturant. The sample matrix of the white wine slightly increased the reaction rate of the system, but the addition to each paraquat standard of the same sample volume as that used for the analysis allowed satisfactory results to be obtained. Table 1 lists the analytical recoveries obtained, which ranged from 89 to 104%.

Conclusions. The results obtained show that the proposed method is a suitable alternative for the simple and fast determination of paraquat. Unlike the enzyme immunoassay methods described for paraquat (Niewola et al., 1985, 1986; Van-Emon et al., 1986; Selisker et al., 1995), which require several incubation steps, the analytical results with this method are obtained in only a few seconds by using a stopped-flow mixing technique, which allows determination of reproducible values of the fast reaction rate of this system and provides a means of accomplishing automation in routine analysis.

The fluorometric detection notably improves the quantification limits achieved in the photometric methods based on the paraquat reduction (Shivhare and Gupta, 1991; Jain et al., 1993; Kuo, 1986; Rai et al., 1997). Although the use of a preconcentration system such as a cation exchange resin has allowed this limit to be decreased (Agudo et al. 1993), this pretreatment step could also be easily applied to the fluorometric method. Finally, with regard to the selectivity of the method, the use of cysteine avoids the interference of diquat, so that its tolerated level is higher than those reported in the aforementioned photometric methods.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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