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Determination of morpholine fungicides using the tris(2,2′-bipyridine) ruthenium(II) chemiluminescence reaction

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Chemiluminescence and electrogenerated chemiluminescence (ECL) methods based on the tris(2,2′-bipyridine) ruthenium(II) chemiluminescence reaction were compared for the analysis of morpholine fungicides. Both methods proved to be sensitive and selective for the determination of dodemorph. In the chemiluminescence system the tris(2,2′-bipyridine) ruthenium(II) was oxidised with Ce(IV) and the flow rate, coil length and pH were optimised by a multivariate method. In the ECL system, the tris(2,2′-bipyridine) ruthenium(II) was oxidised at an aluminium working electrode. The calibration characteristics of the two methods were similar. The linear range was between 2×10^{-7}–1×10^{-5} mol l^{-1} for the chemiluminescence method and 1×10^{-7}–3×10^{-5} mol l^{-1} for the ECL method. The limits of detection were 4.8×10^{-8} mol l^{-1} for chemiluminescence and 4.4 x10^{-8} mol l^{-1} for ECL. A related fungicide tridemorph was also determined by ECL and the linear range for that was between 5×10^{-7} and 5×10^{-5} mol l^{-1}, with a limit of detection of 4.5×10^{-7} mol l^{-1}. An interference study showed that the main interferences for both methods were ascorbic acid and oxalic acid that interfered at the 2×10^{-6} and 1.0×10^{-6} mol l^{-1} level, respectively. Good recoveries (96–100%) were obtained for the determination of dodemorph on cotton gloves and laboratory coats although a methanol extraction was used for the chemiluminescence method and a water extraction for the ECL method. This was because methanol depressed the ECL signal. A study of dodemorph uptake in barley was also carried out. Although ECL was the more elegant method for analysis it was less tolerant to methanol and this could be a disadvantage if it were required to extract the analyte from the sample.

Introduction

Morpholines with large alkyl groups attached, such as dodemorph and tridemorph (Fig. 1), act as systemic fungicides. These fungicides move in the xylem of plants with the transpiration stream and act by inhibiting ergosterol biosynthesis.[1] Analytical methods have been required for their determination when studying uptake by roots and shoots of plants and also when assessing occupational exposure of greenhouse workers.[2] Methods that have been used for their determination include a non-specific coulometric method[3] involving a complex formation with Methyl Orange, a time consuming multi-residue method,[4] gas chromatography,[5] and a radiochemical tracing method.[1] HPLC cannot be used easily because the fungicides will not respond directly to the common sensitive detectors and need to be derivatised.[2] Chamberlain *et al.*[1] studied the uptake by roots and translocation to shoots of dodemorph and tridemorph in barley. They carried out their study using ^{14}C labelled compounds and studied uptake at different pH values by bathing the roots in solutions of different pH. Leenheers *et al*.[5] developed a gas chromatography method with a nitrogen–phosphorous detector when analysing the cotton gloves of greenhouse workers and the foliage dislodgable residue from rose leaves. Their method required time consuming extraction procedures. The limits of detection were 150 μg per pair of gloves and 3 μg per leaf sample. The recovery was over 95% for all samples.

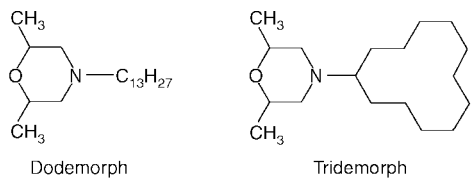

Fig. 1 Chemical structures of dodemorph and tridemorph.

These fungicides contain a tertiary amine functional group (Fig. 1) and therefore they can be determined by using the chemiluminescence reaction with tris(2,2′-bipyridine) ruthenium(II) [$Ru(bpy)_3^{2+}$]. Chemiluminescence occurs when an electronically excited species is produced in a chemical reaction, which emits light on returning to the ground state (direct CL) or alternatively transfers its energy to another molecule which then emits light (indirect or sensitised CL). The technique is very sensitive because measurements are made against a dark background as there is no requirement for a light source.[6] Although the technique is very sensitive it is not inherently selective despite the limited number of chemiluminescence reactions. Selectivity can be introduced by means of separation or sample pre-treatment and consequently, chemiluminesence is often coupled with FIA or HPLC. Flow injection is seen as a particularly good choice for combination with CL detection due to it being an inexpensive technique that provides reproducible and rapid mixing. The analyte is injected into a flowing stream and mixes with the reagent very close to the photodetector. Due to the ease of use and the relatively low cost, the range of analytes that have been detected in this way is vast. These include amines,[7] amino acids,[8] carbohydrates,[9] enzymes,[10,11] vitamins[12] and certain drugs[13] determined by a variety of CL systems.

Chemiluminescence reactions involving $Ru(bpy)_3^{2+}$ can occur in aqueous solution in the presence of dissolved oxygen and other impurities.[14] The reaction is as follows

$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+}$ (oxidation)
$Ru(bpy)_3^{3+} \rightarrow [Ru(bipy)_3^{2+}]^*$ (reduction with analyte)
$[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu$ (chemiluminescence emission)

The oxidation of Ru $(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ can be achieved chemically or by using electrogenerated chemiluminescence (ECL). Electrogenerated chemiluminescence has several advantages over chemiluminescence in that fewer reagents are needed, the applied voltage can control the reaction and the reaction can easily be monitored because it takes place at the electrode surface.[15] The chemiluminescence and electrogenerated chemiluminescence of tris(2,2′-bipyridine) ruthenium(II) and its application has been discussed in great depth in a review by Gerardi *et al.*[16] The reaction has been widely applied to the determination of tertiary amines.[17]

In this work tris(2,2′-bipyridine) ruthenium(II) ECL and chemiluminescence methods are compared for the determination of dodemorph and the ECL activity of tridemorph is briefly investigated. The techniques are then used to determine dodemorph in barley plants and on cotton gloves.

Experimental

Reagents

Tris(2,2′-bipyridine) ruthenium(II) hexahydrate (Pract., 90–95%) was obtained from Fluka (Gillingham, UK). The buffers used contained sodium dihydrogen orthophosphate (AnalaR, 99–102%) from Merck (Poole, UK), sodium acetate (AnalaR, 99%) from BDH (Poole, UK) or sodium carbonate (AnalaR, 99%) from Beecroft and Partners (Rotherham, UK). The pH was adjusted with either sodium hydroxide (analytical-reagent grade, 98%) from Rhône Poulenc (Manchester, UK) or glacial acetic acid (analytical-reagent grade, 99%) from Koch-Light (Haverhill, UK). The sulfuric acid, dodemorph and tridemorph were obtained from Riedel de Haen (Sigma Aldrich, Poole, UK). Cerium(IV) sulfate hydrate was obtained from Merck (Poole, UK). High purity water was produced by reverse osmosis followed by ion exchange (Elgastat UHQ, PSII Elga Ltd., UK) and the reagents used needed no further purification. Stock solutions of dodemorph and tridemorph standards were prepared in methanol because the fungicide was not easily soluble in pure water. Aqueous working standards were then prepared by dilution with water such that the methanol was present at below 1% v/v.

Instrumentation

The ECL instrumentation was the same as has been described previously with the exception of the flow cell.[18] The thin layer flow cell was constructed from two layers of Perspex separated by a 0.3 mm thick PTFE spacer. A channel, 4 mm in width and 30 mm long, was milled into the spacer to provide the flow path. In one Perspex layer, the inlet, working electrode, counter-electrode, reference electrode, and outlet were located, while the other sheet was polished to optical quality. The photomultiplier (PMT) was located above the polished face. A 100 μl sample loop was used and all connections in the flow injection system were constructed from 0.8 mm internal diameter PTFE tubing obtained from Anachem (Luton, Bedfordshire, UK). Solutions were moved through the system with a peristaltic pump with flexible PVC tubing 1.42 mm id (Gilson Minipuls 3, Anachem). Potentials were applied to the electrodes using a three-electrode potentiostat. The electrodes consisted of an aluminium disc working electrode (30 mm^2), platinum-wire counter-electrode and silver pseudo reference electrode which were all housed within the flow cell. The light was detected using a photomultiplier tube (Thorn EMI, 9789QB, Ruislip, UK) and the signals were amplified and recorded using a chart recorder (Chessel, Worthing, Sussex, UK). The high voltage power supply for the PMT was a Model 3000R by Thorn EMI.

The chemiluminescence flow injection equipment was the same as used for ECL. Solutions were delivered to a PTFE T-piece enclosed in a light tight taluminium housing. The reagents were then mixed in a coiled glass flow cell (330 μl) placed directly in front of the PMT. The initial chemiluminescence system on which the optimisation was carried out has three flowing streams, one of dilute sulfuric acid into which the $Ru(bpy)_3^{2+}$ was injected, one of Ce(IV) in dilute sulfuric acid which was mixed with the $Ru(bpy)_3^{2+}$ to oxidise it, and one of dodemorph in buffer. At a later stage the manifold was changed to be more suitable for analysis rather than optimisation, such that the dodemorph could be injected into a buffer stream, rather than forming a continuous stream (Fig. 2). The results for both manifolds were compared using the optimised conditions and no difference was found in performance in terms of linear range and limits of detection.

Sample treatment

Cotton material was spiked with dodemorph to investigate the effect of the matrix and sample preparation on the analytical method. Two 0.5 g pieces of a cotton laboratory coat were treated with 0.68 ml of a solution containing 2.6×10^{-5} mol l^{-1} (4.96 μg) of dodemorph and two 0.8 g pieces of protective cotton gloves were treated with 1.7 ml of a solution containing 2.6×10^{-4} mol l^{-1} (124 μg) of dodemorph. The material was dried at room temperature and the extraction procedure was carried out on these dry pieces. Both water and absolute methanol were investigated as extractants. The fungicide was extracted from the pieces of laboratory coat by shaking them in 10 ml of solvent for 10 min after which the extract could be directly analysed. The portions of cotton gloves were treated similarly but were extracted in 100 ml of solvent.

To measure the uptake of dodemorph in barley plants over 24 h a method was adapted from that described by Chamberlain *et al.*[1] 10 day old plants were transferred to 100 ml of 0.01 mol l^{-1} phosphate buffer containing dodemorph (1×10^{-5} mol l^{-1}). Both pH 5 and pH 8 buffer solutions were investigated. To maintain the pH of the buffer the plants were transferred at 2 h intervals (six changes in all) to fresh solution at the initial dodemorph concentration and pH. After this the plants were moved out of the solution, the shoots and roots were separated and weighed. This gave eight replicated samples at the two pH values from the initial buffer solution, the buffer solution after the treatment and the roots and shoots after treatment. The buffer solutions were analysed directly but it was found necessary to extract the dodemorph by steam distillation from the plant material prior to analysis.

To carry out the steam distillation process the weighed roots or shoots were macerated and then placed in a 250 ml flask for steam distillation. Experiments showed that recoveries of greater than 90% were obtained after 100 ml of distillate was collected, which took approximately 20 min, further distillation was not necessary. Three types of steam distillation experiments were carried out to investigate the effectiveness of the extraction procedure. One type with only 1.0 ml of dodemorph

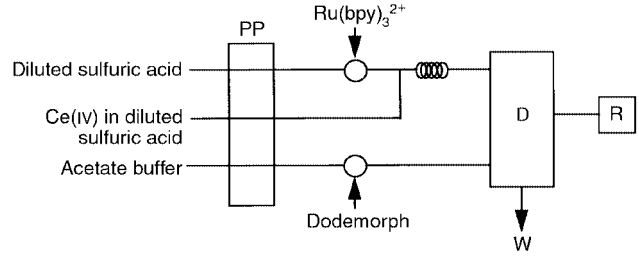

Fig. 2 Flow injection manifold for chemiluminescence detection of dodemorph. PP, peristattic pump; R, readout; W, waste; D, CL detector.

standard (73 μg ml^{-1}) in the flask but no barley samples, one type with the macerated barley samples with no dodemorph present (the blank) and one type with 1.0 ml of dodemorph standard (73 μg ml^{-1}) mixed with the macerated barley samples.

Results and discussion

Optimisation of systems

Chemiluminescence method. For the chemiluminescence study the results of optimisation by both univariate and multivariate approaches were investigated. Ce(IV) was selected as the oxidant as it has been shown to be effective and easy to use.[19] For the univariate study the initial fixed conditions used were H_2SO_4 5×10^{-3} M, Ce(IV) 1×10^{-4} M, $Ru(bpy)_3^{2+}$ 2×10^{-4} M, dodemorph 5×10^{-5} M, total flow rate 5.4 ml min^{-1}, coil length 150 cm, volume injected 45 μl. The PMT was operating at 400 V.

Three buffers were investigated, sodium acetate, sodium dihydrogen orthophosphate and tris(hydroxymethyl)amino-methane (Tris). Sodium acetate was found to be the most effective buffer allowing a high chemiluminesence signal and low blank value. The Tris buffer operated at too acidic a pH range for the method and the phosphate buffer reduced the chemiluminescence signal. To reduce the blank value, lower concentrations of buffer were investigated, but a buffer concentration of 0.5 M was found necessary to provide the buffering capacity needed to control the pH in the system. In Fig. 3 the change in pH value with chemiluminscence can be seen for the most suitable sodium acetate buffer.

The concentration of sulfuric acid was investigated between 5×10^{-3} and 3×10^{-2} mol l^{-1}. The chemiluminescence signal increased with increasing sulfuric acid concentration until a plateau was reached at 2×10^{-2} mol l^{-1}. That concentration was therefore selected for further studies. A similar pattern of increasing chemiluminescence signal with increasing reagent concentration was found for Ce(IV) when it was studied between 9×10^{-4} and 7×10^{-5} mol l^{-1}. The initial increase in gradient was however much steeper and a concentration of 4×10^{-4} mol l^{-1} was selected as appropriate. For $Ru(bpy)_3^{2+}$ the chemiluminescence signal increased as the concentration did and a concentration of 1×10^{-3} mol l^{-1} was chosen as a compromise between signal intensity and consumption of the reagent. The volume of $Ru(bpy)_3^{2+}$ injected was also studied between 35 and 165 μl. The signal initially increased but a plateau was reached at 85 μl, which was the volume used in further experiments.

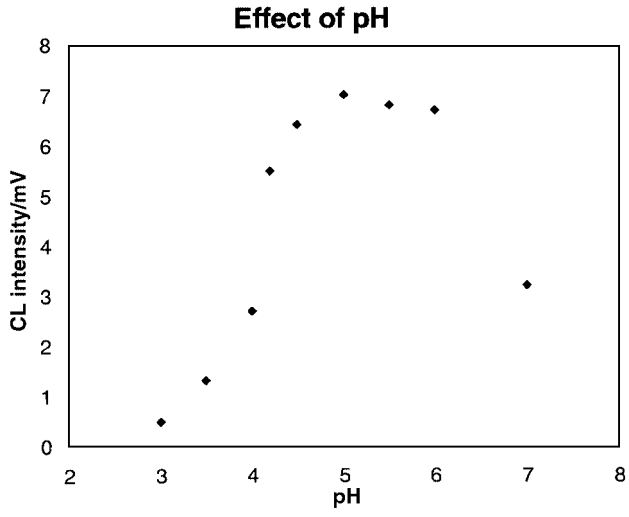

Fig. 3 Effect of pH on the chemiluminescence intensity of dodemorph.

The flow rate, pH and coil length were considered to be important interrelated variables and therefore after an initial univariate optimisation they were optimised by a multivariate method. Yates method of factorial design was used and the high and low levels were set for the three variables using the information gained from the univariate experiments (Table 1). The results of the first experiment showed that increasing the flow rate and pH gave a positive effect but increasing the coil length gave a negative effect. A second set of conditions was then selected, taking into account these results. In the second set of experiments the increase in flow rate had only a small positive effect on the signal and therefore the optimum was near. An increase in pH still had a positive effect on the signal but a lower coil length was found to be necessary. A final set of experiments was then carried out and the optimum flow rate was found to be 9.2 ml min^{-1}, the optimum pH was 6 and the coil length was 40 cm. The results in Table 1 show how the multivariate optimisation favoured a higher flow rate and a lower coil length, giving a lower dispersion of the injected $Ru(bpy)_3^{2+}$. Using the conditions obtained an increase of 200 mV was seen in the chemiluminescence emission signal.

ECL method. A univariate optimisation was carried out for ECL because in this method the FIA system is there to deliver the sample and reagents to the electrode where the reaction is controlled. The inter-related variables effecting dispersion of the sample are therefore less important. The concentration of $Ru(bpy)_3^{2+}$ was set at 1×10^{-3} mol l^{-1} to prevent excessive consumption. One of the most important variables in ECL is the applied voltage and test solutions of 1×10^{-7} mol l^{-1} dodemorph (or tridemorph) with 1×10^{-3} mol l^{-1} $Ru(bpy)_3^{2+}$ prepared in 0.05 mol l^{-1} buffer solution were passed over the working electrode whilst its potential was increased over the range of 0–2 V. The optimum voltage was found to be at +1.33 V for dodemorph and slightly lower at +1.18 V for tridemorph. The pH was investigated between 3.5 and 10 using a range of buffers including sodium acetate, sodium borate and sodium dihydrogen orthophosphate. In Fig. 4 the change in the ECL signal with pH for dodemorph can be seen for sodium dihydrogen orthophosphate and sodium acetate buffer. Sodium dihydrogen orthophosphate buffer was selected as the most appropriate buffer for both compounds but the optimum pH values varied considerably with a pH of 6.5 being selected for dodemorph and pH 9 for tridemorph. The concentration of buffer used was kept as low as possible to ensure low blank values (below 1×10^{-3} mol l^{-1}). The ECL signal was actually found to increase with increasing flow rate with the cell design utilised and therefore a flow rate of 3 ml min^{-1} was selected. This gave a good ECL signal and rapid determinations but did not consume too much reagent.

Calibration characteristics. Using the optimum conditions obtained in the previous section calibration curves were obtained over two decades of concentration corresponding to 1×10^{-7} to 7×10^{-5} mol l^{-1}. Table 2 compares the figures of merit obtained. For the chemiluminescence method a linear range was obtained for dodemorph between 2×10^{-7} and $1 \times$

Table 1 A comparison of univariate and multivariate optimisation for the chemiluminescence method

Variable	Univariate method	Multivariate method
Flow rate/ml min^{-1}	7.1	9.2
pH	5	6
Coil length/cm	60	40

10^{-5} mol l^{-1} where the equation of the line was $y = 34.2 + 1.65 \times 10^7 x$, where y was the chemiluminescence intensity in mV and x was the dodemorph concentration in mol l^{-1}. The correlation coefficient was 0.997. The limits of detection were calculated from the regression line using the method described by Miller and Miller[20] A graph was plotted in the range 0–1.5×10^{-6} mol l^{-1} ($y = 28.1 + 3.04 \times 10^7 x$, correlation coefficient of 0.999) and the limit of detection was calculated to be 4.8×10^{-8} mol l^{-1} (13.5 ng ml^{-1}).

For the ECL method a linear range was obtained between 1×10^{-7} and 3×10^{-5} mol l^{-1} with a correlation coefficient of 0.999. The equation of the line was $y = 3.48 + 3.60 \times 10^7 x$. Again a narrow calibration between 0 and 1×10^{-6} mol l^{-1} was plotted to calculate the limit of detection. These were found to be in close agreement with the chemiluminescence results being 4.4×10^{-8} mol l^{-1} (12.4 ng ml^{-1}). Tridemorph was determined between 5×10^{-7} and 5×10^{-5} mol l^{-1} , the equation of the line was $y = 5.4 + 1 \times 10^7 x$ with a correlation coefficient of 0.999 and a limit of detection of 4.5×10^{-7} mol l^{-1} (134 ng ml^{-1}). Comparing these results with the work by Leenheers *et al.* a lower limit of detection was obtained. Their limit of detection for dodemorph in a leaf was 3 μg and in a pair of gloves was 150 μg, whereas for the ECL method described the limit of detection in a leaf was 2.5 μg and per pair of gloves was 6.8 μg. These comparisons are estimates and are affected by the extraction procedure.

Both the chemiluminescence and electrogenerated chemiluminesence systems showed good reproducibility with RSD values well below 5% in all cases. For example at a concentration of 5×10^{-7} mol l^{-1} the %RSD was 1.67 for the chemiluminescence method.

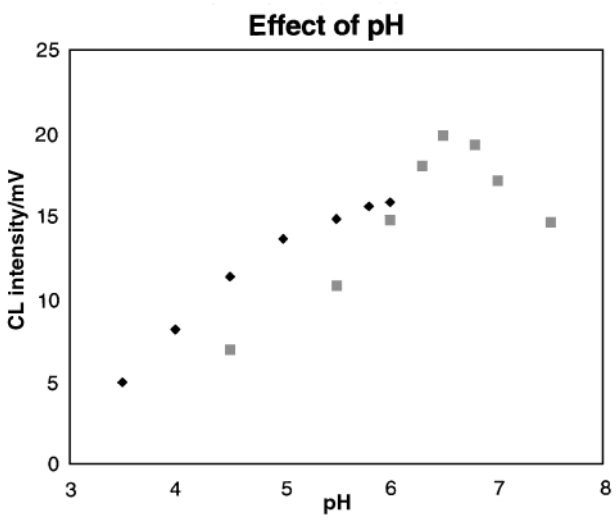

Fig. 4 Effect of pH on the ECL intensity of dodemorph. ◆ Sodium acetate buffer; ▧ sodium dihydrogen orthophosphate.

Interferences

A wide range of different ions and species that could be present in samples such as plants were tested to see if they would interfere with the methods developed for dodemorph. The results of the interference studies can be seen in Table 3. Glucose interfered at the 500-fold level and some interference was seen at the 200-fold level for metal ions such as Fe^{3+} and Cu^{2+}. As would be expected the most severe interferences were seen for ascorbic acid and oxalic acid[14] which were found to interfere at the 2.0×10^{-6} and 1.0×10^{-6} mol l^{-1} level, respectively. Addition of $KMnO_4$ to solutions allowed the concentration that could be tolerated to increase by 5 times. Ascorbic acid and oxalic acid interfere because they are reducing agents and therefore $KMnO_4$, acting as an oxidising agent, alleviates their interferences. Concentrations of up to 1×10^{-5} mol l^{-1} $KMnO_4$ could be used without affecting the ECL.

Applications

Fungicides have been determined in the clothing of greenhouse operatives and in plants such as barley and therefore this type of sample was treated with realistic levels of the fungicide dodemorph to investigate the effectiveness of the method for such matrices.

There was an important difference in the sample preparation methods used for the two different methods. The chemiluminescence method was more tolerant of methanol and therefore methanol could be used for sample extraction. Methanol was found to severely depress any ECL signal if present at greater than the 1% level and therefore an aqueous extraction technique had to be used. As can be seen in Table 4 from the results comparing water and methanol extraction, the recovery is the same within experimental error. A slightly higher recovery was seen for ECL but this was still acceptable within experimental error.

Table 2 Figures of Merit

Method	Compound	Calibration equation	Correlation coefficient	Linear range/mol l^{-1}	%RSD range ($n = 5$)	LOD/ mol l^{-1}
CL	Dodemorph	$y = 1.65 \times 10^7 x + 34.2$	0.997	2×10^{-7} ~ 1×10^{-5}	1.07 ~ 3.98	4.8×10^{-8}
ECL	Dodemorph	$y = 3.60 \times 10^7 x + 3.48$	0.999	1×10^{-7} ~ 3×10^{-5}	1.12 ~ 4.2	4.4×10^{-8}
ECL	Tridemorph	$y = 1.00 \times 10^7 x + 5.4$	0.999	5×10^{-7} ~ 5×10^{-5}	0.89 ~ 3.78	4.5×10^{-7}

Table 3 Interference study

	Maximum tolerant concentrations/mol l^{-1} (recovery 95 ~ 106%)		
Interferences	CL method for 5×10^{-7} mol l^{-1} dodemorph	ECL method for 1×10^{-6} mol l^{-1} dodemorph	ECL method for 2×10^{-6} mol l^{-1} tridemorph
Na^+, K^+, NH_4^+, Cl^-, NO_3^-, SO_4^{2-}, CO_3^{2-}	5×10^{-4}	1×10^{-3}	2×10^{-3}
Ca^{2+}, Mg^{2+}, Al^{3+}, Zn^{2+}, PO_4^{3-}, glucose	2.5×10^{-3}	5×10^{-3}	5×10^{-3}
Fe^{3+}, Cu^{2+}, Ni^{2+}	1×10^{-4}	1×10^{-4}	2×10^{-4}
Co^{2+}	1×10^{-4}	5×10^{-5}	5×10^{-5}
Ascorbic acid	5×10^{-7}	2×10^{-6} (1×10^{-5})[a]	2×10^{-6} (2×10^{-5})[a]
Oxalic acid	5×10^{-7}	1×10^{-6} (5×10^{-6})[a]	1×10^{-6} (1×10^{-5})[a]

[a] In the presence of 1×10^{-5} mol l^{-1} $KMnO_4$.

Table 4 The recovery of dodemorph from cotton matrices

Solvent	Dodemorph added/μg	Dodemorph found/μg		Recovery (%)	
Lab coat—		CL	ECL	CL	ECL
Methanol	4.96	4.95	—	99.7	—
Water	4.96	4.88	5.15	98.4	103.8
Gloves—					
Methanol	124	122.0	—	98.3	—
Water	124	119.2	120.5	96.1	97.2

Table 5 Uptake of dodemorph in barley plants

Sample	CL method (%)	ECL method (%)
pH 8—		
Solution	81.40	82.39
Leaves	2.99	2.93
Roots	0.37	0.35
pH 5—		
Solution	80.0	80.48
Leaves	0.7	0.61
Roots	0.01	0.092

Table 5 shows the results obtained for the study of the uptake of dodemorph in barley shoots. Severe unidentified interferences were seen for the analysis of the roots and shoots giving poor recoveries. The only way this could be overcome was by carrying out a steam distillation. The experimental agreement between the ECL method and the chemiluminescence method was excellent. The experimental results show that the uptake of dodemorph into the plant is greater in more alkaline conditions (pH 8).

Conclusion

The chemiluminescence techniques utilised for the determination of fungicides have been shown to be sensitive and selective. The performance characteristics of the methods are directly comparable. The experimental procedure for ECL is simpler and more elegant with oxidation of the $Ru(bpy)_3^{2+}$ occurring at the electrode. This advantage is however lost if the samples to be analysed need to be extracted in methanol because the ECL signal then becomes severely depressed. Both methods were affected by unknown interferences in the plant material and sample preparation by steam distillation was necessary before good recoveries could be obtained.

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