# Electrogenerated Chemiluminescent Determination of Pyruvate Using Tris(2,2′-bipyridine)ruthenium(II)*

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**A novel method for the sensitive and selective determination of pyruvate is presented, using an electrogenerated chemiluminescence (ECL) reaction of tris(2,2′-bipyridine)ruthenium(II) in the presence of cerium(III) nitrate, increasing the number and diversity of analytes currently detected by this ECL reaction. Various experimental conditions have been optimized, leading to a calibration over three-orders of magnitude of concentration, with a detection limit of $3.1 \times 10^{-7}$ mol $l^{-1}$ (27 ppb). Simple methods have also been developed to suppress the interference effects of various compounds that are expected to be present in potential samples from fermentation media.**

***Keywords:*** *Electrogenerated chemiluminescence; tris(2,2′-bipyridine)ruthenium(II); pyruvate*

## Introduction

Pyruvate is a biologically important molecule involved in a variety of biochemical reactions in plants and animals, either as a substrate, product or intermediate. The measurement of pyruvate concentrations can, therefore, give valuable information as to the progress of specific biochemical reactions. For example, the assay of the activity of many enzymes is determined by monitoring the accumulation or depletion of pyruvate levels. In a medical context the determination of pyruvate can assist in the diagnosis of some diseases. For example, raised levels of pyruvate in cerebral spinal fluid are observed in patients with pyruvate dehydrogenase deficiency and other brain abnormalities.[1]

The determination of pyruvate in the brewing industry can also be a useful measurement. Lactic acid bacteria have a more prominent role than yeasts in the metabolism of organic acids in wine- and beer-making; however, in the presence of sub-optimum concentrations of nitrogen compounds yeast cells produce high levels of pyruvate.[2] Oxygen-containing acids are important in fermentation because they are able to bind $SO_2$, thus lowering the concentration of free $SO_2$, which is necessary for the safe preservation of wine. One of the most important products from the metabolism of pyruvate by lactic acid bacteria is diacetyl, a compound with a low aroma threshold that can spoil beer but improve wine flavour.[3] Flavour maturing processes in other foods, *e.g.*, onions, can also lead to changes in pyruvate concentrations, which can be correlated to flavour perception.[4]

Electrogenerated chemiluminescence (ECL) is emerging as a useful new tool for the determination of a variety of analytes. ECL is a technique by which a chemiluminescent reaction is produced in the vicinity of an electrode, by active species generated electrochemically. Whilst retaining the advantages of sensitivity and selectivity inherent to conventional chemiluminescence (CL) methods, producing the CL reaction by electrical stimulation allows greater control over the initiation, rate and course of the reaction. ECL also allows CL methods to be simplified using flow systems with reduced numbers of reagents, since active reagents can be produced electrochemically from passive precursors in the sample or carrier stream. In addition it is often possible to regenerate reagents, and occasionally analytes, allowing the ECL signal to be sustained for longer than conventional CL, and each molecule can emit several photons per measurement cycle; factors which enhance the sensitivity of the technique.

The analytical usefulness of this technique has been the subject of recent reviews.[5,6] As a result it is clear that the most promising and rapidly developing area of analytical ECL concerns the reactions of tris(2,2′-bipyridine)ruthenium(II), [$Ru(bpy)_3^{2+}$]. This is because the reactive complex $Ru(bpy)_3^{3+}$ is readily regenerated electrochemically after its light-producing reaction with the analyte which forms the excited species $Ru(bpy)_3^{2+*}$, and such reactions occur in aqueous buffered solutions in the presence of oxygen and other impurities. The analytically useful reactions involving this complex fall into four main groups; firstly, the determination of a wide variety of mainly tertiary, and some secondary, aliphatic amines. This has been used for the analysis of drugs,[7,8] amino acids,[9,10] antibiotics[11–13] and reduced nicotinamide adenine dinucleotide (NADH),[13] and shows potential for use in determining an extensive range of tertiary amine pharmaceuticals and agrochemicals; secondly, the determination of oxalate in vegetables[14] and biological samples;[15] thirdly, the use of labels based on $Ru(bpy)_3^{2+}$ for polymerase chain reaction (PCR) analysis,[16] and immunoassay,[17] by reaction of the label with added tripropylamine; and fourthly, the selective determination of peroxodisulfate.[18]

This paper describes a new analytical method based on a reaction of $Ru(bpy)_3^{2+}$, for the determination of pyruvate. The ECL reaction between $Ru(bpy)_3^{2+}$ and organic acids including pyruvic acid was first proposed and demonstrated by Rubinstein and Bard in 1981.[19] In the present work flow injection ECL methodology has been developed and the reaction and experimental conditions optimized, to allow the sensitive and selective determination of pyruvate to low limits of detection. Other organic acids and various interfering compounds that would be expected to be present in biological samples have been examined for their interference effects on the determination of pyruvate, and chemical methods for the removal or suppression of those compounds causing detrimental effects have been developed.

## Reaction of Tris(2,2′-bipyridine)ruthenium(II) With Oxalate and Pyruvate

It is useful to firstly examine the mechanism established for the ECL reaction of $Ru(bpy)_3^{2+}$ with oxalate,[19] used for the

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determination of the latter. In this work the reaction has been modified to enable the determination of the structurally similar pyruvate anion.

Oxalate is irreversibly oxidized to $CO_2$ at platinum electrodes within the platinum oxide region. This oxidation is known to be completely suppressed on an oxide-covered platinum electrode surface;[20] however, on such an electrode the reversible $Ru(bpy)_3^{2+/3+}$ couple is readily observed.[19] Hence, in the determination of oxalate the following reaction is used. Firstly, the $Ru(bpy)_3^{2+}$ is oxidized at the electrode to the $Ru(bpy)_3^{3+}$ cation. This species is then capable of oxidizing the oxalate ($C_2O_4^{2-}$) in the diffusion layer close to the electrode surface to form an oxalate radical anion ($C_2O_4^{\bullet -}$). This breaks down to form a highly reducing radical anion ($CO_2^{\bullet -}$) and carbon dioxide. The reducing intermediate then reduces more of the $Ru^{3+}$ complex back to the parent complex in an excited state, which emits light ($Ru(bpy)_3^{2+*}$). ($\lambda_{max}$ = 620 nm).

The oxidation of pyruvate, is also suppressed by an oxide layer on the platinum electrode surface. In this case, unlike oxalate, the $Ru(bpy)_3^{3+}$ ion is not sufficiently oxidizing to oxidize the pyruvate in solution, and hence the ECL reaction does not proceed. It is possible, however, to add a more powerful oxidizing agent to the solution to initiate the oxidation of the pyruvate, and hence the ECL reaction. Not wishing to complicate the procedure or instrumentation for the carriage and mixing of reagents by the addition of a strong oxidizing agent, this is best performed by the addition of non-reactive cerium(III) to the sample solution containing $Ru(bpy)_3^{2+}$ and pyruvate in 0.03 mol $l^{-1}$ sulfuric acid. By applying a higher positive potential to the electrode, the active oxidant cerium(IV) can be formed *in situ*. This species can then oxidize the pyruvate, which reacts to form the strongly reducing intermediate $CH_3CO^{\bullet}$. This species behaves in a similar way to $CO_2^{\bullet -}$, and participates in electron-transfer reactions with the $Ru(bpy)_3^{3+}$, analogous to the reactions observed for oxalate, to produce chemiluminescence.[19] The reaction mechanism is best summarized and visualized in Fig. 1.

## Experimental

### *Instrumentation*

Fig. 2 shows the layout of the flow injection system and instrumentation used for the electrogeneration of chemiluminescence. A carrier stream of 0.03 mol $l^{-1}$ dilute sulfuric acid is constantly pumped through the manifold, into which sample volumes (100 μl) are injected and carried into the flow cell, over the electrodes and out to waste. Since the ECL reaction is only initiated at the electrode surface, all the reagents could be combined with the sample solution prior to injection into the manifold with no resulting reaction, hence eliminating the need for any in-line mixing of reagents. This procedure was useful in the method development stage; however, when implementing future applications it is envisaged that the integration of the sample with reagent solutions will be carried out on-line. The flow cell is fabricated from a solid PTFE block encapsulated by a light-tight closely fitting aluminium casing. The flow cell consists of an elliptical spacer sandwiched between a clear glass observation window, and a back-plate housing the disc electrodes. The reaction is initiated at a platinum-disc working electrode (4 mm diameter). Also housed within the cell is a silver-disc pseudo-reference electrode (4 mm diameter), and a platinum-wire counter electrode downstream of the main flow cell. Although silver metal is not normally considered adequate as a reference electrode for electrochemical measurements, as it can be easily polarized and is hence subject to drift, it has proved to be sufficiently stable throughout the experiments. It also offers the advantages of simplicity, small size, ease of construction and minimum solution contamination. Potentials are applied to the electrodes by a computer-controlled three-electrode potentiostat designed and built in-house. The light produced is detected by a photomultiplier tube (PMT) (9789 QB, Thorn EMI, Ruislip, UK), which is held at 1000 V by a high-voltage power supply (Model 3000R, Thorn EMI). The signals obtained are amplified and recorded as a flow injection peak on a chart recorder (Chessel, Worthing, UK).

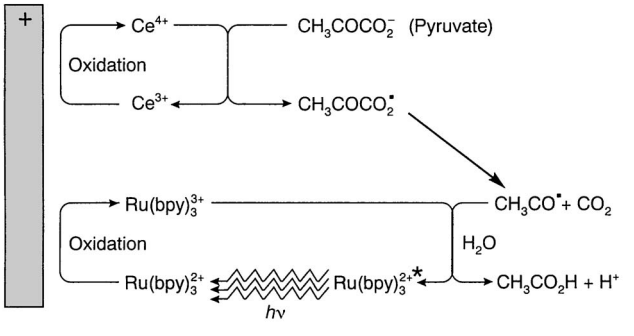


**Fig. 1** ECL reaction of pyruvate with $Ru(bpy)_3^{2+}$ in the presence of cerium(III).

### *Reagents*

Tris(2,2′-bipyridine)ruthenium(II) hexahydrate (Pract., 90–95%), cerium(III) nitrate hexahydrate (Puriss, >99%), and sodium pyruvate (Puriss, >99%) were all obtained from Fluka (Gillingham, UK). Sulfuric acid (2.5 mol $l^{-1}$ AnalaR) for dilution, was obtained from Merck (Poole, UK). Iron(III) nitrate nonahydrate (general purpose reagent) was obtained from Hopkin and Williams (now Merck), and all other compounds used in the interference study were of analytical-reagent grade. All reagents were stored in dry conditions over calcium chloride, and used without further purification. All standard and sample solutions were made up with water prepared by reverse osmosis followed by ion exchange (Elgastat UHQ, PSII Elga, High Wycombe, UK.)

## Results and Discussion

### *Effect of Applied Voltage*

Volumes of sample solution (10 μl) containing $2 \times 10^{-4}$ mol $l^{-1}$ pyruvate, $1 \times 10^{-3}$ mol $l^{-1}$ $Ru(bpy)_3^{2+}$ and $5 \times 10^{-3}$ mol $l^{-1}$ cerium(III) nitrate in 0.05 mol $l^{-1}$ $H_2SO_4$, were injected into a carrier stream of 0.05 mol $l^{-1}$ $H_2SO_4$, and hence passed over the working electrode, which was held at a set of linearly increasing positive potentials. The ECL intensity, measured as

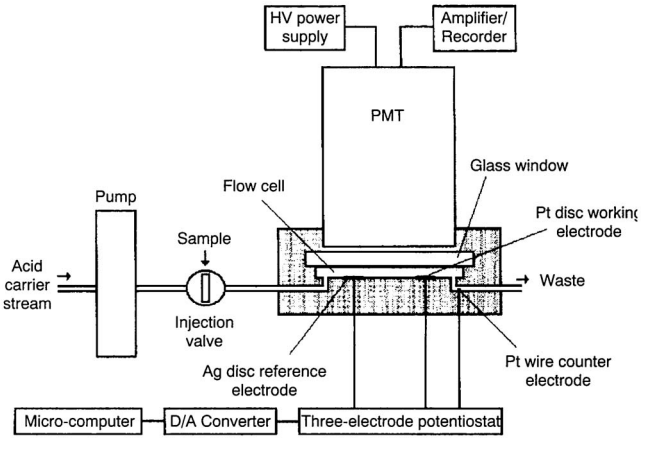


**Fig. 2** Schematic diagram of the instrumentation used for the electrogeneration of chemiluminescence.

an average peak height, is plotted *versus* the applied voltage in Fig. 3. Each point is the mean of four replicate injections. The optimum voltage was observed to be +1.55 V (*versus* silver-metal pseudo-reference electrode), slightly higher than the oxidation potential observed for the cerium$^{3+/4+}$ couple measured under similar experimental conditions. This voltage was used throughout the remainder of the study.

### *Effect of Sulfuric Acid Concentration*

The medium used for this ECL reaction had to fulfil several criteria. The medium should (*i*) be able to maintain a reasonably stable low pH (less than pH 2), to maintain the solubility of cerium(IV) the active oxidant whilst not forming an insoluble salt with either cerium(III) or (IV); (*ii*) not react with cerium(IV); (*iii*) not contain R–CH(OH)COOH or R–COCOOH compounds, that are likely to take part in analogous ECL reactions (*i.e.*, a citrate buffer); and (*iv*) not be electrochemically active under the experimental conditions used.

Dilute sulfuric acid fulfils these criteria and hence was selected for the study. The concentration of acid used was optimized by injecting a range of sample solutions containing 2 $\times$ 10$^{-4}$ mol l$^{-1}$ pyruvate, 5 $\times$ 10$^{-3}$ mol l$^{-1}$ cerium(III) nitrate and 1 $\times$ 10$^{-3}$ mol l$^{-1}$ Ru(bpy)$_3^{2+}$ in 0–0.50 mol l$^{-1}$ $H_2SO_4$, into a corresponding carrier stream of the same concentration $H_2SO_4$.

The results are shown in Fig. 4, with each point being the mean of four replicate injections. A sharply defined optimum range centred on 0.03 mol l$^{-1}$ $H_2SO_4$ was observed. The ECL intensity decreases at higher acid concentrations. The cause of this has not yet been fully determined, but is thought to be due to undesirable side reactions of the radical intermediates with hydrogen ions present at high concentrations. At sulfuric acid concentrations lower than 0.03 mol l$^{-1}$ a rapid decrease in ECL peak height is observed with consecutive injections. This observation was accompanied by a build up of a thin yellow film on the electrode surface which could only be removed by polishing, which led to the suggestion that cerium(IV) is being deposited onto the electrode surface which blocks the ECL reaction. A similar effect is observed when using a weaker acid, such as ethanoic acid, as the carrier medium.

The analytical consequence of the sharp optimum observed for sulfuric acid concentration is that relatively minor variations in this concentration would cause significant variation in the test signals. However, provided the acid carrier stream and sample diluting solution are accurately prepared, at the concentration of sulfuric acid used, variations in the sample pH were observed to have a negligible effect on the pH of the final solution, and hence ECL intensity.

### *Effect of Cerium(III) Concentration*

Cerium(IV) was selected as the oxidant for the reaction due to the inactivity of the oxidant precursor (3+ form), the high oxidizing strength of the oxidized species, and for the simplicity and ease of the electrochemical oxidation–reduction reaction cycles. The effect of the concentration of the cerium(III) reagent was determined by injecting solutions of 2 $\times$ 10$^{-4}$ mol l$^{-1}$ pyruvate, 1 $\times$ 10$^{-3}$ mol l$^{-1}$ Ru(bpy)$_3^{2+}$ and 0–0.075 mol l$^{-1}$ cerim(III) nitrate in 0.03 mol l$^{-1}$ $H_2SO_4$ into a carrier stream of 0.03 mol l$^{-1}$ $H_2SO_4$. The results (see Fig. 5) show that the ECL intensity increases with increasing cerium(III) concentration, reaching a maximum level at 0.005 mol l$^{-1}$, which is 25 times the concentration of the pyruvate analyte. Each point is the mean of four replicate injections. The ECL intensity then remains approximately constant up to a concentration of 0.075 mol l$^{-1}$ cerium(III). Higher concentrations were not tried. Hence, an excess of cerium(III) was used for pyruvate determinations, and a concentration of 7.5 $\times$ 10$^{-3}$ mol l$^{-1}$ was selected for use throughout the remainder of the study.

### *Effect of* Ru(bpy)$_3^{2+}$ *Concentration*

For the Ru(bpy)$_3^{2+}$ reaction with pyruvate the ECL intensity of the signal and blank injections is increased by increasing the concentration of Ru(bpy)$_3^{2+}$, up to millimole per litre levels. However, owing to the expense of the reagent it is desirable to limit its excessive consumption. Hence a concentration was selected to give reasonable intensity and measurable signals for the sample and blank solutions. This was selected as 1 $\times$ 10$^{-3}$ mol l$^{-1}$ for this study.

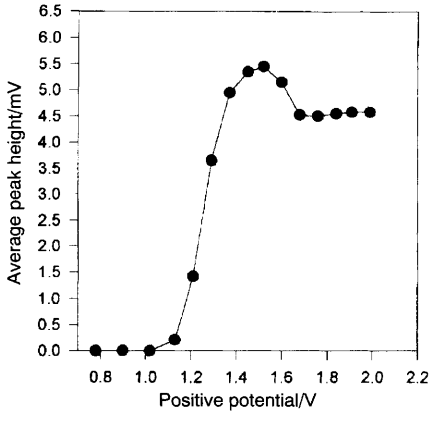


**Fig. 3** Variation of ECL intensity with applied positive potential.

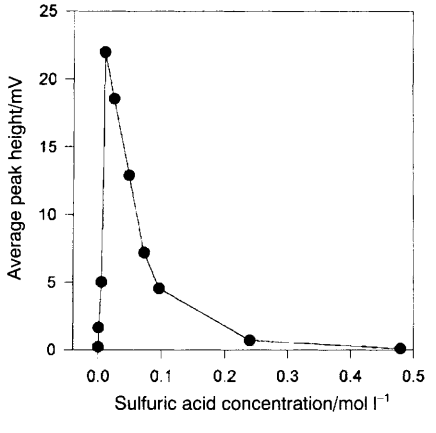


**Fig. 4** Variation of ECL intensity with sulfuric acid concentration.

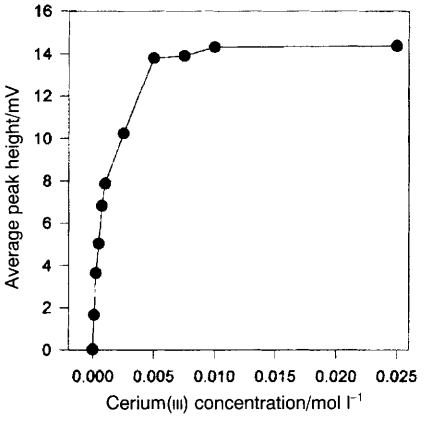


**Fig. 5** Variation of ECL intensity with cerium(III) concentration.

### *Effect of Flow Rate*

A test solution containing $2 \times 10^{-4}$ mol $l^{-1}$ pyruvate and the optimum concentration of reagents was injected into the acid carrier stream, which had a flow rate in the range 0.60–2.05 ml $min^{-1}$, and the ECL intensity measured. The peak heights were found to be approximately constant across this range of flow rates, and the reproducibility of the measurements remained high ($s_r < 1.5\%$, $n = 4$) in each case. Hence, the ECL reaction is thought to be sufficiently rapid as to not be perturbed by flow rates up to 2.05 ml $min^{-1}$, the maximum attainable with the pump used. A pump speed of 1.70 ml $min^{-1}$ was selected to give a fast sample throughput time with high reproducibility. The sample throughput is currently 60 sample injections per hour.

### *Calibration Characteristics*

Using the optimum conditions: reagents, $1 \times 10^{-3}$ mol $l^{-1}$ $Ru(bpy)_3^{2+}$, $7.5 \times 10^{-3}$ mol $l^{-1}$ cerium(III) nitrate in 0.03 mol $l^{-1}$ $H_2SO_4$, with a carrier stream of 0.03 mol $l^{-1}$ $H_2SO_4$, a flow rate of 1.7 ml $min^{-1}$, and + 1.55 V applied to the working electrode; a log–log calibration was achieved over three decades of concentration (see Fig. 6). Each point on the graph is the mean of five replicate injections, and the $s_r$ of the measurements ranged from 0.44 to 3.21% with an average of 1.84%.

The limit of detection (LOD) was determined by plotting on a linear scale the last six points of the calibration ($0–20 \times 10^{-6}$ mol $l^{-1}$ pyruvate), and using this graph to assign the LOD as the concentration of pyruvate giving rise to a signal equal to the blank plus three times the standard deviation of the blank. The linear plot has a correlation coefficient of 0.99995 ($n = 6$). The LOD was estimated to be $3.1 \times 10^{-7}$ mol $l^{-1}$, equivalent to 27 ppb. Both calibration graphs are shown in Fig. 6.

These results can be compared with other methods of analysis. For example pyruvate can also be determined colorimetrically by reaction with 2,4-dinitrophenylhydrazine, although the method is non-specific. Hence pyruvate is most frequently and more sensitively and selectively determined enzymically. This is performed by measuring the reduction in concentration of NADH as it is transformed to $NAD^+$ during the conversion of pyruvate to lactate by lactate dehydrogenase. The concentration of NADH can be determined spectrophotometrically by absorption or fluorescence. The ECL method was observed to be more sensitive than the absorbance method, with a LOD lower by an order of magnitude, and similar to that obtained by enzymic analysis with fluorescence detection.[21]

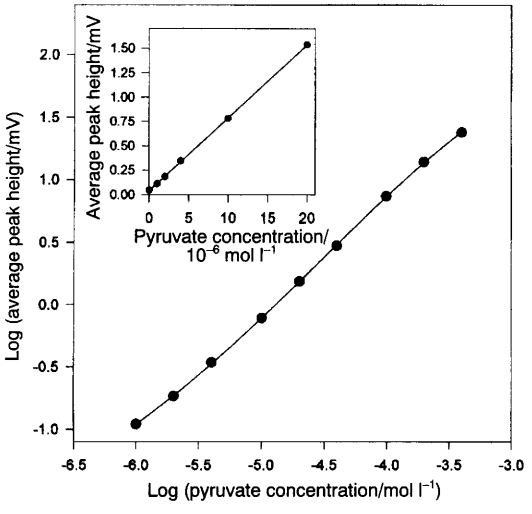


**Fig. 6** Log–log and linear calibration (inset) graphs for pyruvate.

The enzymic method does not suffer from phosphate interference (see later), and hence requires less sample pretreatment. However, most biological sample extracts will contain material with fluorescence bands which overlap with that of the reduced pyridine nucleotides, and hence produce background fluorescence. Such species should not effect the ECL method, which is based on more specific CL reactions. Also the ECL method is more readily adapted to flow injection methodology, and is convenient and reproducible, with a higher sample throughput, by a factor of three, than the enzymic method.

### *Interferences*

The interference study was designed to include a range of other organic acids to assess the selectivity of the technique, and other compounds that are likely to be present in samples derived from fermentation media, a potential application of the technique. Such compounds include phosphate buffer, and trace amounts of vitamins and EDTA commonly used in brewing media.

### *Organic Acids*

A survey of other organic acids was carried out to see which compounds would produce an ECL signal under the optimum experimental conditions for the determination of pyruvate, and hence give rise to positive interference. Compounds were selected with either R–CH(OH)COOH or R–COCOOH functional groups or multiple carboxylic acid groups. The ECL responses relative to a pyruvate standard are shown in Table 1. As expected, the most significant interferent is oxalate, which will react with $Ru(bpy)_3^{3+}$ even in the absence of cerium(IV). However the ECL response obtained for oxalate is lower than would be observed in the absence of cerium(III) due to the limited solubility of cerium(III) oxalate. If oxalate were to be present in a sample, then an ion-exchange sample clean-up for example would be needed. Citrate and EDTA also produced significant interference signals. Citrate is an α-hydroxy acid of the form R–CH(OH)COOH; however, EDTA contains multiple acid groups and is a ditertiary amine, which are both characteristics that could give rise to an ECL response. The exact mechanism of the ECL reaction of EDTA has not been determined. The suppression of citrate and EDTA is discussed later. The other organic acids tested give low responses and pose no significant interference problems.

### *Amines*

The optimum pH for $Ru(bpy)_3^{2+}$ ECL reactions used in the determination of a range of tertiary and secondary amines, ranges from about pH 5–7. At low pH the amines are protonated and give rise to only very low ECL responses. Hence it is postulated that tertiary and secondary amine compounds will not produce significant interference signals since the reaction media is about pH 1.55. Preliminary investigations using a range of vitamin compounds have supported this conclusion.

**Table 1** Relative ECL signals from a range of organic acids

| Compound (as sodium salt) | Concentration/ mol $l^{-1}$ | Relative ECL signal (%) |
|---|---|---|
| Pyruvate | $2 \times 10^{-4}$ | 100.0 |
| Oxalate | $2 \times 10^{-4}$ | 113.4 |
| Citrate | $1 \times 10^{-3}$ | 34.1 |
| Malonate | $1 \times 10^{-3}$ | 4.8 |
| Tartrate | $1 \times 10^{-3}$ | 3.7 |
| Lactate | $1 \times 10^{-3}$ | 1.5 |
| EDTA | $1 \times 10^{-4}$ | 14.0 |
| Blank | — | 0.3 |

### *Suppression of the Interferents, EDTA and Citrate, With Iron(III)*

Since EDTA and citrate are both multidentate ligands chelating through oxygen atoms, they readily form strong complexes with iron(III) ions, which are stable in acid solution. Complexation with iron(III) ions were observed to substantially reduce the interference signal from these compounds, whilst only slightly suppressing the signal for pyruvate, with which iron(III) only forms a weak complex. The effect of the addition of iron(III) to test solutions of pyruvate, EDTA and citrate is shown in Fig. 7. Iron(III) is an ideal masking agent for these compounds due to its electrochemical inactivity under the experimental conditions used. By complexing with the highly positively charged iron(III) ion these compounds are potentially less prone to oxidation by the similarly positively charged cerium(IV) ion.

It is speculated that other organic acids that have multiple carboxylic acid groups, and pose a possible ECL interference problem, may also be masked by the use of iron(III).

### *Phosphate Interference*

The presence of phosphate in the sample solution was observed to quench the ECL reaction. For example, for a solution of $2 \times 10^{-4}$ mol $l^{-1}$ pyruvate, the signal obtained is reduced to just 6.5% of the original signal on the addition of $4.5 \times 10^{-3}$ mol $l^{-1}$ potassium dihydrogenorthophosphate. The suppression was thought to be due to the formation of a complex between the phosphate and cerium(III) or (IV) ions. The further addition of excess of acid or cerium(III) did not significantly improve the signals obtained in the presence of phosphate. In the acidic conditions used cerium(III) is not precipitated in phosphate concentrations up to 0.015 mol $l^{-1}$.

Since the $pK_a$ values of pyruvate and phosphate are very similar, 2.49 and 2.12, respectively, a method of ion-exchange sample pre-treatment is not suitable. Hence, wishing to minimize sample preparation, the simplest solution is to use precipitation. This can be performed using the cerium(III) reagent already required in solution for the ECL reaction, without the need for the addition of extra reagents. If excess of cerium(III) is used and the solution is adjusted to pH 6.0 with concentrated sodium hydroxide solution, all of the phosphate is precipitated from solution as the cerium(III) salt, and can be removed by centrifugation and decantation. The sample is then adjusted to the low pH required for the ECL reaction by the addition of $H_2SO_4$, to a concentration of 0.03 mol $l^{-1}$. This procedure does not precipitate the pyruvate analyte.

## Conclusions and Future Work

The method described herein has been shown to be suitable for the analysis of pyruvate in acidic solutions, over a wide concentration range and to a low limit of detection (27 ppb). The method also shows high reproducibility and repeatability. Interference signals arising from other organic acids and the suppression of the ECL by phosphate ions has been successfully overcome, and applications for the technique are envisaged in the biotechnology field. Preliminary results have shown that this method is suitable for the analysis of samples extracted from the fermentation of a brewers yeast after removal of the interfering phosphate by the simple method of cerium(III) precipitation described. The results compared well with a conventional enzymic method.

Future work will be concerned with the application of the technique to a variety of real samples, and investigations will be made into the possibility of immobilizing the $Ru(bpy)_3^{2+}$ and a suitable co-oxidant, which can be re-cycled at the electrode and hence produce a self-contained flow-through sensor for on-line applications. The possibility of on-line precipitation/separation methods for the removal of phosphate are also being considered.

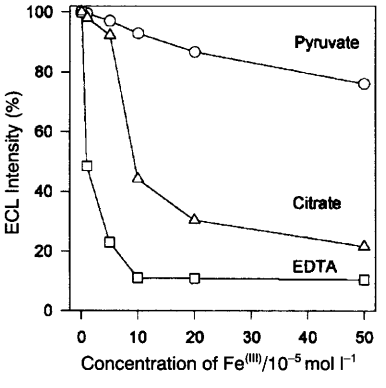


**Fig. 7** Effect of the addition of iron(III) on the ECL intensity (as a percentage of the original signal) of pyruvate, EDTA and citrate. (Concentration of the analytes, $1 \times 10^{-4}$ mol $l^{-1}$.)

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