Electrocatalytic Tetracycline Oxidation at a Mixed-Valent Ruthenium Oxide—Ruthenium Cyanide-Modified Glassy Carbon Electrode and Determination of Tetracyclines by Liquid Chromatography with Electrochemical Detection

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Mixed-valent films of ruthenium oxide—ruthenium cyanide were electrodeposited onto glassy carbon and characterized for the electrocatalytic oxidation of tetracycline. The currents produced by tetracycline were higher than from previously reported electrode modifications or pre-treatments. In H₂SO₄ pH 1.0 from previously reported electrode modifications or pre-treatments, the Ru(III/IV) couple of ruthenium oxide was 3 × 10⁵ ± 1 × 10⁵ mol⁻¹ cm⁻³ s⁻¹, and the rate of charge diffusion through the films was 4.5 × 10⁻⁷ ± 3.5 × 10⁻⁷ cm² s⁻¹. Reaction was localized at the film–solution interface. When used as detectors in liquid chromatography (in H₂PO₄ pH 2.5 × 0.1 M KH₂PO₄ + 20% CH₃CN, E = 1.10 V vs SCE), the electrodes gave limits of detection (~3 S/N) of 0.1 ppm for tetracycline and oxytetracycline and 0.5 ppm for doxycycline and chlorotetracycline. These limits were suitable for FDA and Codex Alimentarius guidelines for tetracyclines in food. Recoveries of the four tetracyclines from sea and freshwater shrimp were in the range 73–111%, which was higher or similar to the previously reported recoveries from shrimp.

The monitoring of drugs used in food industries such as livestock, poultry production, and aquaculture is of growing interest worldwide, due to concern that overuse can lead to bacteria developing antibiotic resistance. Tetracycline (TC) and derivatives such as oxytetracycline (OTC), chlorotetracycline (CTC), and doxycycline (DC) are probably the most widely used antibiotics in aquaculture and are also used in nutrition and feed additives throughout the agricultural sector. Hence, there is a growing need to monitor tetracyclines in the food industry.

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Standard methods for tetracycline analysis usually employ microbiologically based procedures. However, these are time-consuming and expensive. Also they can only determine the total antibiotic content of a sample, not the identity or quantity of the individual antibiotics present. Recently, more rapid and selective methods have been developed using liquid chromatography (LC) with UV or fluorescence spectrophotometry detection. A further improvement has been to use either mass spectrometric or electrochemical detection. Since these methods generally provide higher selectivity and sensitivity than UV or fluorescence. Between the two, electrochemical systems can be implemented at considerably lower cost.

Tetracycline has previously been oxidized at Au and Pt electrodes but was shown to poison both surfaces, although in the case of Au, some reaction was still possible at the oxide, after the metal had been blocked. Carbon has been found to be a better surface for tetracycline oxidation, and carbon fiber micro-

2266 Analytical Chemistry, Vol. 76, No. 8, April 15, 2004 10.1021/ac035085b CCC: $27.50 © 2004 American Chemical Society Published on Web 03/06/2004
electrodes have been used for OTC oxidation at 1.6 V versus Ag wire.\textsuperscript{24} Glassy carbon has been used as an amperometric LC detector at 1.2 V versus Ag/AgCl.\textsuperscript{25} It was shown that electrochemical pretreatment in phosphate buffer could improve the electrode response, but periodic repolishing and retreating was needed to prevent the sensitivity from going down.\textsuperscript{8} An alternative, and potentially more stable way to improve the response, is to modify the glassy carbon with a redox couple. This has been done using the oxhydroxide, NiO(OH), which reacted with tetracycline.\textsuperscript{8} We found in preliminary experiments (unpublished) that ruthenium oxide in carbon paste was catalytic to tetracycline in a similar manner. However, oxide and oxhydroxide films are not practical for tetracycline analysis in foods, since they are only stable in an alkaline pH, whereas in the analysis of meat or seafood, an acidic pH is needed to digest the proteins that would otherwise bind to tetracycline and prevent its extraction.\textsuperscript{5}

A technique for using ruthenium oxide electrocatalysis in acidic solutions is to electodeposit mixed-valence films of ruthenium oxide and hexacyanoruthenate, as first described by Kulesza\textsuperscript{19} and designated here as mvRuO–RuCN. Films of mvRuO–RuCN have been shown to be adherent on glassy carbon and catalytic to a number of substances, including aliphatic alcohols,\textsuperscript{20} methionine,\textsuperscript{21} insulin,\textsuperscript{22} hydrazine,\textsuperscript{23} glutathione,\textsuperscript{21} arsenic(III),\textsuperscript{24} cysteine,\textsuperscript{25} aliphatic and furanic aldehydes,\textsuperscript{26} thiocyanate,\textsuperscript{27} and N-nitrosamines.\textsuperscript{28} This suggests they may be used to oxidize the alcohol groups present on tetracycline and its derivatives. Hence, the objective of this report is to examine the kinetics of tetracycline oxidation at mvRuO–RuCN films and to evaluate the detection of tetracycline and some of its derivatives in real samples, using liquid chromatography with a mvRuO–RuCN-based detector. Shrimp were chosen as the real samples, from consideration of the steadily increasing amount of shrimp produced by aquaculture (rising from \(\sim 100\) million lb worldwide in 1979 to 1600 million lb in 1999),\textsuperscript{29} from the resistance already developing in human and shrimp pathogens in southeast Asia toward some of the antibiotics (including tetracyclines) used in shrimp farms,\textsuperscript{30} and from the relatively small number of chromatographic studies that have examined shrimp as a sample matrix.\textsuperscript{30,31}

**EXPERIMENTAL SECTION**

Ruthenium salts, tetracycline (TC), and the derivatives chlorotetracycline hydrochloride (CTC), doxycycline hydrochloride (DTC), and oxytetracycline hydrochloride (OTC) were provided by Sigma/ Aldrich. Solutions were prepared using distilled water.

A standard three-electrode cell was employed, with a 3-mm diameter glassy carbon working electrode (area 0.0962 cm\(^2\)), Pt wire counter, and SCE reference. Potential control and data collection were performed with an Autolab PGSTAT 10 with GPES software version 4.3. Collector—generator experiments employed the bipotentiotstat module of the Autolab, with a glassy carbon ring-Pt disk working electrode supplied by EG&G. Before each experiment, the glassy carbon electrode was polished with an alumina—water slurry on cotton wool and then sonicated in distilled water.

Mixed-valence ruthenium oxide/ ruthenium cyanide films were prepared by essentially the same procedures as those previously reported,\textsuperscript{26} i.e., by cycling in a solution of \(\text{H}_2\text{SO}_4\) at pH 2.0, containing 0.5 M NaCl + 2 mM RuCl\(_3\) + 2 mM \(\text{K}_2\text{Ru(CN)}\)\(_6\), the only difference being that the potential was swept (50 mV s\(^{-1}\)) from \(-0.2\) to 1.2 V instead of to 1.1 V. This was because we observed mvRuO–RuCN films would grow quicker when cycled to 1.2 V. The possible changes to the mvRuO–RuCN electrochemistry are discussed in the Results section. The number of scans applied depended on the film thickness requirement and was typically between 5 and 100.

The electrochemistry of the mixed-valence films was examined in \(\text{H}_2\text{SO}_4\) at pH 1.0 containing 0.5 M \(\text{K}_2\text{SO}_4\). Before each experiment, a cyclic potential of 0.4–1.2 V was repeatedly applied to the modified electrodes at 5 mV s\(^{-1}\) in the working electrolyte until stable voltammograms were obtained.

LC experiments were performed using a Shimadzu series LC-10AD solvent delivery system, equipped with a Reheodyne model 7125 injector and a 20\(\mu\)L sample loop, in conjunction with a BAS electrochemical cell. Separation was achieved with a 4.0 mm × 100 mm octadecylsilane column (C18) having a 5-\(\mu\)m particle size. The mobile phase was 20%acetonitrile (unless otherwise stated) in 0.1 M \(\text{KH}_2\text{PO}_4\) adjusted to pH 2.5 with \(\text{H}_2\text{PO}_4\). Oxygen was removed from the mobile phase by sparging helium into the flow stream for a few minutes. The flow rate was 1.5 mL/min unless otherwise stated.

Sample preparation for determining tetracycline recoveries was as follows: Sea- and freshwater shrimp were purchased locally. Sections of shrimp tissue were cut into small pieces and ground. Samples of \(\sim 2\) g were weighed out accurately and then added to conical flasks containing 10 mL of 0.1 M \(\text{H}_2\text{PO}_4\). Duplicate flasks of seawater shrimp were spiked with a combination of OTC, CTC, and DTC, each at 20 ppb. Two unspiked flasks were used as blanks. In the same manner, duplicate flasks of freshwater shrimp were spiked with the same tetracyclines, each at 2 ppb. Again, two flasks were left unspiked as duplicate blanks. After adding 10 mL of acetonitrile, each flask was left to stand for 10 min, and the contents were then passed through a filter paper (Whatman No. 5). A 5-\(\mu\)L aliquot of each filtrate was then placed in a separation funnel. After adding 2.5 mL of hexane and 2.5 mL of dichloromethane, the mixture was shaken and left to separate out. The lower (aqueous) layer was removed, centrifuged at 4000 rpm, and then filtered using a 2.5-\(\mu\)m filter cartridge of 0.45-\(\mu\)m pore size (Millipore). The resulting filtrate was injected into the flow stream.

Optical measurement and film observation was made with an Olympus BX40F-3 microscope.
RESULTS AND DISCUSSION

Cyclic Voltammetry of mvRuO–RuCN Films. Figure 1 shows a cyclic voltammogram (50 mV s$^{-1}$) for the deposition of a mixed-valence ruthenium film in pH 1.0 sulfuric acid. The voltammetry agrees with that previously observed, namely, broad reduction/oxidation peaks across approximately 0.05 to −0.2 V, for the Ru(II/III) couple of ruthenium oxide, an anodic peak at −0.8 V for the conversion of Ru(II) to Ru(III) in the hexacyanoruthenate, an anodic peak at −1.0 V for the conversion of Ru(III) to Ru(IV) in the ruthenium oxide, and a cathodic peak at −0.7 V for the reduction of both the previously generated Ru(IV) oxy and Ru(III) cyano species. Using the charge under the cathodic peak to estimate coverage, it was found that varying the number of deposition scans from 5 to 100 varied the amount of immobilized ruthenium from $0.2 \times 10^{-7}$ to $2.3 \times 10^{-7}$ mol cm$^{-2}$. The ruthenium peaks were well-defined in pH ≤3.0. It has been suggested that this is because an acidic medium is needed to stabilize the cationic ruthenium oxo species.

The cathodic peak current at 0.7 V was noted at varying scan rates, $v$, for a film grown from 20 deposition scans. For $v \sim 30$ mV s$^{-1}$, the peak current scaled linearly with $v$ (Figure 1, inset b) but showed slight curvature with $v$ (Figure 1, inset a) (see Supporting Information). This is the characteristic of a predominantly surface reaction, in which most redox species are contacting the electrode. However, the Ru coverage for this film, calculated from the charge under the cathodic peak, was found to be $1.9 \times 10^{-9}$ mol cm$^{-2}$, which assuming $10^{-28}$ mol cm$^{-2}$ for a Ru monolayer, must actually be in the order of 190 monolayers. The fact that the characteristics of a surface reaction are still observed in the voltammetry suggests the rate of charge diffusion in the films is fast. This was also suggested by amperometric and ring–disk experiments, as described later.

Tetracycline Electrocataylsis at mvRuO–RuCN. As noted in the introduction, it is envisaged that tetracycline would be determined in an acidic medium following deproteinization. Therefore, the reaction of tetracycline was examined at the mixed-valence films at pH 1.0. Figure 2 (main) shows a slow scan rate voltammogram (5 mV s$^{-1}$) of the modified electrode in the absence and presence of 0.25 mM tetracycline. In the presence of tetracycline, a decrease in the reduction peak and an increase in the 1.0 V oxidation peak is observed, indicating oxidation of tetracycline mediated by the Ru(III/IV) couple of ruthenium oxide.

As noted in the Experimental Section, the films used here were grown by scanning the potential up to 1.2 V, rather than 1.1 V as in previous works. Hence, the tetracycline oxidation may have involved surface ruthenium oxo groups of oxidation states above IV, as previously indicated for RuO$_2$ electrodes. The oxidation peak at 1.0 V in the absence ($I_c$) and presence ($I_d$) of 0.25 mM tetracycline was measured as in Figure 2 (main), as a function of Ru coverage, for a series of films grown by cycling up to either 1.1 or 1.2 V. As shown in the inset to Figure 2, $I_c/I_d$ decreases with increasing film thickness, as expected, as the reacting Ru sites form a smaller proportion of the total coverage. However, it can be seen that the characteristics of the decrease are similar and the $I_c/I_d$ values for both sets of films lie on a common curve. This suggests little difference between the two deposition potentials, in terms of the Ru species participating in the reaction.

We examined the rate of the tetracycline–Ru(IV) reaction using the cyclic voltammetry theory of Andrieux and Saveant. They have shown that, for monolayer coverage of a redox catalyst (i.e., where concentration polarization can be neglected), the

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Figure 1. Cyclic voltammogram of mvRuO–RuCN film deposition (20th scan). Conditions as given in the Experimental Section. Scan rate 50 mV s$^{-1}$. Inset a: change in Ru coverage of two different films, during repetitive scanning (50 mV s$^{-1}$) in Ru-free solutions of either H$_2$SO$_4$ pH 1.0 + 0.5 M K$_2$SO$_4$ (C) or 20% CH$_3$CN/H$_3$PO$_4$ pH 2.5 + 0.1 M KH$_2$PO$_4$ (E). Inset b: scan rate dependence of cathodic peak at 0.7 V for mvRuO–RuCN film grown by 20 scans. Electrolyte is H$_2$SO$_4$ pH 1.0 + 0.5 M K$_2$SO$_4$. All experiments were performed at room temperature (20 ± 3 °C).

Figure 2. Cyclic voltammogram of mvRuO–RuCN film in H$_2$SO$_4$ pH 1.0 + 0.5 M K$_2$SO$_4$ in the absence (dotted line) and presence (solid line) of 0.25 mM tetracycline. Scan rate is 5 mV s$^{-1}$. Inset: ratio of oxidation peak height in the presence ($I_c$) and absence ($I_d$) of 0.25 mM tetracycline as a function of Ru coverage, for films grown by cycling up to either 1.1 (C) and 1.2 V (E). Scan conditions as in main figure.

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current at the electrode can be related to the concentration, \( c \), of the substrate by

\[
I_p = \text{const} nF A c \sqrt{D_n F \nu / R T}
\]  

(1)

where \( I_p \) is \( I_e - I_s \) and the other symbols have their usual significance. The “constant” in eq 1 increases as a function of \( \log(kT/\text{D}_n \nu / R T) \)\(^{12} \) as shown in the working curve of ref 30, where \( k \) is the second-order rate constant for the mediator—substrate reaction and \( I \) is the mediator coverage. Hence, for a given system, the “constant” calculated from eq 1 can be related to \( k \) via the working curve.

The value of \( D \), for the diffusion of tetracycline in the bulk solution, was estimated from the Wilkie-Chang correlation:\(^{35} \)

\[
D_{AB} = 1.173 \times 10^{-6} T \sqrt{\frac{Q_M}{h_0 V_A^{0.6}}}
\]  

(2)

where \( D_{AB} \) is the diffusion coefficient of solute A in solvent B. \( M_B \) is the molecular weight of solvent B, \( M_A \) is the viscosity of B, \( V_A \) is the solute molar volume, calculated as 0.06 m\(^3\)kg\(^{-1}\)mol\(^{-1}\) from the table in ref 35, and \( Q \) is an “association parameter”, which is 2.6 for water.

We attempted to grow monolayer films on the electrode by applying deposition scans to a more dilute solution of the film components, i.e., 0.025 M NaCl + 0.1 mM K\(_2\)Ru(CN)\(_6\) + 0.1 mM RuCl\(_3\). It was not possible to achieve coverage by one monolayer only. However, as observed by optical microscopy, we were able to coat the electrode fully with loadings of \( \sim 30 \) monolayers. Using the previous relation that one monolayer represents a coverage of \( 10^{-10} \) mol cm\(^{-2}\)^{19} if we take each Ru center as corresponding to occupation of a cube-shaped volume, then from Avogadro’s number, one monolayer has a thickness of \( 1.3 \) nm. Hence, 30 monolayers corresponds to \( \sim 40 \) nm, although given the granular morphology of some Ru systems,\(^{36} \) this estimation of thickness is quite approximate. However, for films of up to \( \sim 1500 \) nm, it can be reasonably assumed that concentration polarization is not significant.\(^{37} \)

Therefore, we applied the method of Andrieux and Saveant to films of \( \sim 30 \) monolayers, at 5 MV sec\(^{-1}\), in the absence and presence of 0.25 mM tetracycline. Duplicate experiments with different films gave \( k = 3 \times 10^9 \pm 1.0 \times 10^9 \) mol cm\(^{-1}\) cm\(^2\) sec\(^{-1}\).

**Steady-State Tetracycline Oxidation at mvRuO–RuCN.** Tetracycline was oxidized amperometrically at 1.10 V, using a film grown by 20 deposition scans. The resulting calibration, shown in Figure 3, had a sensitivity of 2.59 A M\(^{-1}\) cm\(^{-2}\) and a limit of detection (2 × signal/noise) of 68 nM (i.e., 0.03 ppm). The previous method of tetracycline oxidation using NiO(OH)\(^{38} \) produced a sensitivity of 0.12 µA m\(^{-1}\) cm\(^{-2}\), which for the electrode area reported (0.47 cm\(^2\)) is 0.113 A M\(^{-1}\) cm\(^{-2}\). Hence, the use of mvRuO–RuCN films produced a considerably higher response.

Varying the Ru coverage by \( \sim 1 \) order of magnitude did not significantly change the amperometric response to 1.2 µM tetracycline (see Supporting Information). Variation of the cover-


Figure 3. Steady-state current for tetracycline oxidation at 1.10 V using film grown by 20 scans. Electrolyte as in Figure 2.
measuring the coverage of the films using CV, the potentials of the ring and disk were held at 0.40 and 1.10 V, respectively, until steady-state currents were obtained, as shown in Figure 4. The width of the gap, found by optical microscope prior to coating, was 1.5 x 10^{-2} cm. For the range of coverages applied (1.7 x 10^{-1}-3.1 x 10^{-1} mol cm^{-2}), the film thicknesses were in the range 2.2 x 10^{-4}-4.0 x 10^{-4} cm. Hence, the polymer layers were much less than the gap distance. Under these conditions, linear concentration gradients can be assumed across the gap for the osmium(II/III) conversion in the cyanoruthenate and Ru(III/IV) in the ruthenium oxide. The measured film thicknesses were in the range 2.2 x 10^{-7} cm.-2, which suggests virtually no difference in cvRuCN films. It has been found by Lyons et al. to be 4.2 x 10^{-9} cm^2 s^{-1} for Ru(bpy)_2(PVP)_2Cl (bpy = 2,2'-bipyridyl, PVP = poly(4-vinylpyridine)) and 1.9 x 10^{-9} cm^2 s^{-1} for polyaniline,41 and by Chen et al. to be 8.5 x 10^{-7} cm^2 s^{-1} for Os(II/III) in PSS-PVP (PSS = polystyrene sulfonate) and 1.5 x 10^{-8} cm^2 s^{-1} for Os(II/III) in Naion40, both groups using the same collector-generator method. Hence, the mvRuO-RuCN films provide high rates of charge transfer, approaching those of Os(II/III) in PSS-PVP. The high rates are indicated in the value of the "characteristic current", I_E, for charge diffusion, calculated from the measurement of four different films gave D_E = 4.5 x 10^{-7} ± 3.5 x 10^{-7} cm^2 s^{-1}. As far as we know, D_E has not previously been measured for mvRuO-RuCN films. It has been found by Lyons et al. to be 4.2 x 10^{-9} cm^2 s^{-1} for Ru(bpy)_2(PVP)_2Cl (bpy = 2,2'-bipyridyl, PVP = poly(4-vinylpyridine)) and 1.9 x 10^{-9} cm^2 s^{-1} for polyaniline,41 and by Chen et al. to be 8.5 x 10^{-7} cm^2 s^{-1} for Os(II/III) in PSS-PVP (PSS = polystyrene sulfonate) and 1.5 x 10^{-8} cm^2 s^{-1} for Os(II/III) in Naion40, both groups using the same collector-generator method. Hence, the mvRuO-RuCN films provide high rates of charge transfer, approaching those of Os(II/III) in PSS-PVP. The high rates are indicated in the value of the "characteristic current", I_E, for charge diffusion, calculated from

\[ I = 2\pi nF\Gamma_D / \delta \]  

(5)

where r_D is the disk radius, \( \delta \) is the gap width, and D_E can be considered the electron diffusion coefficient in the film and is a measure of the rate of charge precoolation. It should be noted that the experiment here involves a steady state being reached for two separate one-electron-transfer reactions, namely, the Ru(II/III) conversion in the cyanoruthenate and Ru(III/IV) in the ruthenium oxide. The measured D_E is therefore an overall value, representing a combination of the rates of electron self-exchange for both these processes.

M measurements of four different films gave D_E = 4.5 x 10^{-7} ± 3.5 x 10^{-7} cm^2 s^{-1}. As far as we know, D_E has not previously been measured for mvRuO-RuCN films. It has been found by Lyons et al. to be 4.2 x 10^{-9} cm^2 s^{-1} for Ru(bpy)_2(PVP)_2Cl (bpy = 2,2'-bipyridyl, PVP = poly(4-vinylpyridine)) and 1.9 x 10^{-9} cm^2 s^{-1} for polyaniline,41 and by Chen et al. to be 8.5 x 10^{-7} cm^2 s^{-1} for Os(II/III) in PSS-PVP (PSS = polystyrene sulfonate) and 1.5 x 10^{-8} cm^2 s^{-1} for Os(II/III) in Naion40, both groups using the same collector-generator method. Hence, the mvRuO-RuCN films provide high rates of charge transfer, approaching those of Os(II/III) in PSS-PVP. The high rates are indicated in the value of the "characteristic current", I_E, for charge diffusion, calculated from

\[ I_E = nFAD_E / L^2 \]  

(6)

as 9 mA, in comparison with 0.1 \mu A for I_E calculated from eq 3. Comparison of eqs 4 and 6 gives D_{Ru} \sim 5 x 10^{-6} cm^2 s^{-1}.

**Tetracycline Oxidation in CH_3CN/H_3PO_4.** The measurement of TC and three derivatives, OTC, CTC, and DC, was performed by liquid chromatography using a mvRuO-RuCN-modified glassy carbon electrode as the detector (at 1.10 V). The mobile phase was a mixture of phosphoric acid and acetonitrile at pH 2.5. This pH value was chosen from consideration of the stability of both the mixed-valence film (stable, pH \leq 3) and the column, (Si eluted at pH \leq 2). The presence of acetonitrile in the mobile phase was to improve the mobile-phase selectivity. Increasing the acetonitrile content of the mobile phase from 5 to 20% increased the response to tetracycline. Beyond 20%OTC and TC were not separated, and therefore, 20% was used for all further experiments.

To assess the effect of changing electrolyte, we recorded the steady-state current to 1.2 \mu M tetracycline in CH_3CN/H_3PO_4 at pH 2.5, as a function of Ru coverage. The response was again thickness independent, but with currents \sim 4.8 times greater than in H_2SO_4 at pH 1.0 (see Supporting Information). For a fast rate of charge transfer, thickness independence means the system is in the "SR" case of Andrieux et al.\(^ {38}\) In this condition, an increased response can come from increases in (1) substrate partition into the film, (2) substrate diffusion through the film, and (3) the rate constant for tetracycline oxidation. Changes in (1) and (2) were examined by recording CVs of given films at pH 2.5, in both H_2SO_4 and CH_3CN/H_3PO_4, each case in the absence of supporting electrolyte. Changes in film swelling sufficient to increase the rate of substrate partition/diffusion can be expected to also increase the ionic concentration in the diffuse layer. From Gouy-Chapman theory, this will increase the diffuse layer capacitance.\(^ {40}\) Since the ionic concentration is low, the diffuse layer capacitance will control the overall differential capacitance, C_D.\(^ {41}\) The value of C_D was determined at a potential in the cyclic voltammograms where no faradaic current occurred (E = 0.520 V), using C_D = I/v. Two films of 4.3 x 10^{-4} and 4.2 x 10^{-4} mol cm^{-2} Ru coverage gave C_D = 0.35 and 0.36 mF, respectively, in H_2SO_4 and 0.38 and 0.37 mF in CH_3CN/H_3PO_4, which suggests virtually no difference in film swelling. This indicates a negligible change in the value of D_E. However, we note that tetracycline is less soluble in 20%CH_3CN than in pure H_2O (e.g., 15 mg of tetracycline could be dissolved by 150 \mu L of H_2SO_4 but needed 2.05 mL of CH_3CN/H_3PO_4). Hence, the partition coefficient of tetracycline into the films will be higher from CH_3CN/H_3PO_4 than from H_2SO_4.

Changes in (3) were examined by measuring the ratio I/E (v = 5 mV s^{-1}) for the oxidation of 0.25 mM tetracycline. The same film used at pH 1.0 gave I/E = 1.7 in H_2SO_4 but 1.3 in CH_3CN/H_3PO_4. A different film used in H_2SO_4 gave I/E = 1.5 at pH 1.0 and 1.6 when transferred to pH 2.5. Similarly, a film used in CH_3CN/H_3PO_4 gave I/E = 1.1 at pH 1.0 and 1.2 at pH 2.5. Hence, there is some sensitivity to the change in anion, with PO_4^3- producing lower catalytic activity to tetracycline. However, this effect is exceeded by an increase in catalytic activity caused by

\[ \text{Tetracycline Oxidation in CH}_3\text{CN/H}_3\text{PO}_4. \]
the increase in pH. This is reasonable, since the tetracycline reaction is believed to occur via oxidation of a phenol group, and will hence generate protons, and so become more favorable at higher pHs.

Film stability in the two electolytes was compared by noting the Ru coverage during repetitive potential cycling ($\nu = 50 \text{ mV s}^{-1}$). As shown in inset a of Figure 1, the films show greater stability in H$_2$SO$_4$.

Tetracycline Measurement by Liquid Chromatography.

The elution of the four tetracyclines, using H$_3$PO$_4$ containing CH$_3$CN at 20%, is shown in Figure 5. It should be noted that the resolution at this sensitivity might be improved further by using a CH$_3$CN gradient in the mobile phase.

Calibrations of the four tetracyclines are summarized in Table 1. The limits of detection (3 $\times$ signal/noise ratio) were 0.1 ppm for TC and OTC and 0.5 ppm for DC and CTC. These values are not as low as some of the reported LC methods using MS detection, which have generally achieved ppb or sub-ppb levels. The FDA does not approve tetracyclines for aquaculture (unlike the EU and Asia). However, FDA guidelines allow tetracyclines in cattle, dairy calves, swine, sheep, chickens, turkeys, catfish, lobsters, and salmonids and state that the total residues should be below 2 ppm for muscle content and may be higher for organs such as liver or kidneys. The Codex Alimentarius Commission of the FAO/WHO advises tetracycline concentrations for a number of animal food products in the range 0.1–0.6 ppm, depending on the tissue. Hence, although the detection limits here are not as low as for MS methods, they show that in many cases less costly electrochemical systems can be adequate LC detectors for tetracyclines in foods.

Figure 6 shows the response to 30 additions of 10 ppm OTC at mvRuO$_2$–RuCN electrode (•), pretreated bare electrode (○), and untreated bare electrode (●), using LC. Flow rate 1.5 mL/min; other conditions as in Figure 5.

Table 2. Recoveries of Tetracyclines from Shrimp Samples by LC

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<th>recovery (%)</th>
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<td>CTC at 2 ppm</td>
<td>17.5</td>
<td>1.6</td>
<td>80.0</td>
</tr>
<tr>
<td>CTC at 2 ppm</td>
<td>16.9</td>
<td>1.5</td>
<td>75.0</td>
</tr>
<tr>
<td>DC at 20 ppm</td>
<td>85.3</td>
<td>20.6</td>
<td>103.0</td>
</tr>
<tr>
<td>DC at 20 ppm</td>
<td>90.9</td>
<td>22.1</td>
<td>110.5</td>
</tr>
<tr>
<td>DC at 2 ppm</td>
<td>12.1</td>
<td>1.5</td>
<td>75.0</td>
</tr>
<tr>
<td>DC at 2 ppm</td>
<td>11.9</td>
<td>1.5</td>
<td>75.0</td>
</tr>
</tbody>
</table>

Concentrations spiked at 20 ppm were from seawater shrimp and that at 2 ppm from freshwater shrimp.

Figure 5. LC separation of TC, OTC, CTC, and DC (all at 5 ppm) with detection at mvRuO$_2$–RuCN electrode poised at 1.10 V. Mobile phase 20% CH$_3$CN in 0.1 M KH$_2$PO$_4$/H$_3$PO$_4$ pH 2.5. Flow rate was 1.0 mL/min for elution of OTC and TC and 1.5 mL/min for CTC and DC.

Table 1. Calibration of Tetracyclines by LC

<table>
<thead>
<tr>
<th>antibiotic</th>
<th>sensitivity (µA ppm$^{-1}$ cm$^{-2}$)</th>
<th>$r^2$</th>
<th>LOD (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>0.343</td>
<td>0.9932</td>
<td>0.1</td>
</tr>
<tr>
<td>OTC</td>
<td>0.343</td>
<td>0.9932</td>
<td>0.1</td>
</tr>
<tr>
<td>CTC</td>
<td>0.066</td>
<td>0.9934</td>
<td>0.5</td>
</tr>
<tr>
<td>DC</td>
<td>0.040</td>
<td>0.9955</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Flow rate 1.5 mL/min; other conditions as in Figure 5. Curve plotted from seven concentrations, five injections at each concentration.

Analytical Chemistry, Vol. 76, No. 8, April 15, 2004 2271

before determining each concentration recovered. The results are shown in Table 2. So far, most LC determinations of tetracyclines in foods have concentrated on either cattle, milk, or animal feed samples. To the best of our knowledge, there have only been two examinations of tetracycline in shrimp. In one, a range of tetracyclines were recovered at ∼50% and in the other, OTC was recovered at 77%. Hence, the protocol described here is similar or better than previous methods.

CONCLUSIONS
Films of mvRuO–RuCN on glassy carbon have high conductivities and show relatively high reaction rates in oxidizing tetracyclines. Hence, the current at the modified electrodes is thickness independent (which may be useful for fabrication) and is controlled by the rate constant for tetracycline oxidation, partition of tetracyclines into the films, and diffusion through them. The presence of CH$_3$CN in the electrolyte increased the response, by increasing the partition coefficient of tetracycline into the films, but it also lowered film stability. Increasing the pH increased the response by increasing the rate constant for tetracycline oxidation.

The sensitivities for tetracycline detection at mvRuO–RuCN films were higher than previous methods using either bare or modified electrodes. When used as detectors in liquid chromatography, the mvRuO–RuCN electrodes showed limits of detection adequate for regulatory requirements. The method of sample pretreatment produced recoveries from shrimp similar or higher than those previously obtained.

ACKNOWLEDGMENT
This project was funded by BIOTEC (grant code BT-B-07-2F-B5-302). M.S. acknowledges a BIOTEC research fellowship.

SUPPORTING INFORMATION AVAILABLE
Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review September 16, 2003. Accepted February 6, 2004.
AC035085B