Principles of EC Detection and Troubleshooting Guide

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MF-9083 INSTRUCTION MANUAL

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MANUFACTURER'S NOTE

The instruments and products described in this manual, either wholly or in part, are manufactured for research purposes only. Use for medical diagnosis is not intended, implied or recommended by the manufacturer. Use for this purpose and accountability for the same rests entirely with the user.

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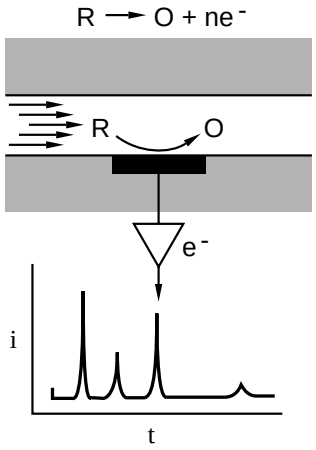
Section 1. Principles of Electrochemical Detection

This section includes basic background information on the theory of electrochemical detection. It is not mathematically oriented. Concepts important to the LCEC technique are explained. Bioanalytical Systems commercialized these concepts in 1974. While the instrumentation has evolved a great deal since then, the basic principles remain the same.

1.1 Electrochemical Fundamentals

LCEC may be considered in terms of electrolysis at a fixed point along a flowing stream. The stream in this case is the eluent from the analytical LC column and a sequence of solute ("analyte") zones separated with varying degrees of resolution. These zones pass into a very low-volume thin-layer cell, where the flow is constrained to a thin film passing over a planar electrode held at a fixed potential. Figure 1.1 illustrates an exploded view of this region. If the potential is greater (more positive for oxidation, more negative for reduction) than that required for the electrolysis of the analyte, a measurable charge passes from electrode to analyte (or vice versa). The resulting current is directly proportional to the concentration of solute passing through the channel.

Figure 1.1. Exploded view of electron transfer at the surface of a thin-layer electrode. A laminar flow profile passes over the electrode in the thin-layer zone containing reduced analyte R. Oxidation to O at the electrode surface releases electron(s) to the surface. This current is subsequently converted to a voltage, which drives the recorder and produces the chromatogram.



The electrode may be thought of as a chemical reagent. The more positive its potential, the stronger an oxidizing agent it becomes; when the potential is made more negative, it becomes a stronger reducing agent. In either case, as the concentration of solute rises and falls in passing through the thin-layer cell, the electrolysis current proportionately follows these changes. This current, as a function of time, is amplified and sent to a recorder to yield a chromatogram.

LCEC is an AMPEROMETRIC determination. Unlike measurements (such as pH) of a potential difference under zero-current conditions, LCEC measures current at a fixed potential. It must always be remembered that this experiment involves heterogeneous electron transfer (from one phase to a different phase), and the success of the experiment depends in large part upon the care with which these components are chosen and operated.

Crucial to all amperometric determinations is FARADAY'S LAW, which states that:

$$Q = nFN \qquad (1)$$

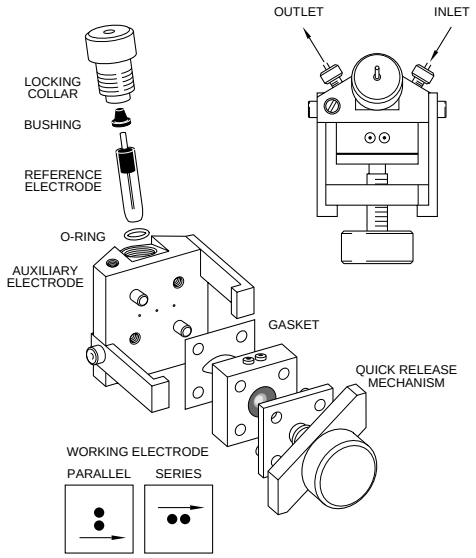
Q is the number of coulombs (a unit of charge) used in converting N moles of material, n is the number of moles of electrons lost or gained in the transfer process per mole of material, and F is Faraday's constant (96,500 coulombs/mole of electrons). Differentiation of (1) with respect to time yields the current, which is the measure of the rate at which material is converted:

$$\frac{dQ}{dt} = i = nF\frac{dN}{dt} \qquad (2)$$

Equation (2) therefore relates a measurable quantity, the current, to the fundamental redox process occurring in the cell.

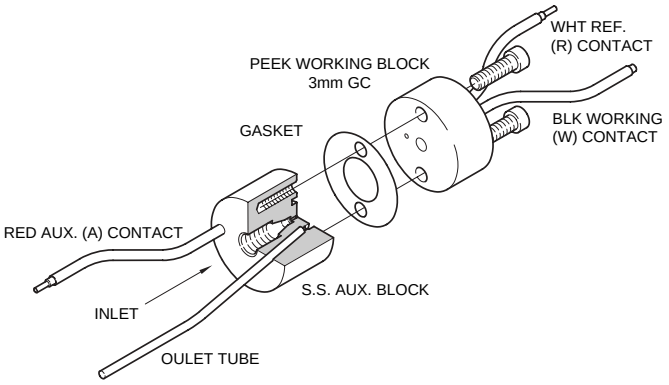
Bioanalytical Systems established a new standard for its cross-flow electrochemical transducers in the BAS 200A and BAS 480 liquid chromatography systems (Figure 1.2). As from the beginning, the proven thin-layer concept remains central to this design. In it, two blocks form a sandwich around a thin fluoropolymer gasket which defines a microliter flow-cell. Two working electrodes (glassy carbon or other material) are embedded along one wall of the channel, whereas the reference and auxiliary electrodes are directly opposite, typically only 50 micrometers distant.

Figure 1.2. Cross-flow thin layer cell for LCEC.



In 1994, BAS introduced the UniJet cell (Figure 1.3), specifically for microbore LCEC. The UniJet utilizes a radial flow thin-layer cell which has some advantages in low-flow, low-dead-volume situations.

Figure 1.3. UniJet cell design for BAS microbore chromatography.



The nature of the working electrode, particularly its bulk composition and surface treatment, is critical to detector performance. Many organic compounds react at significantly different rates depending on the electrode used. It is normally desirable to carry out the electrode reactions at the greatest possible rate in order that the current be limited only by mass transport of molecules to the surface and not by their reaction rate at the surface. This situation affords the greatest sensitivity and stability without sacrificing selectivity.

Carbon paste (a mixture of spectroscopic graphite powder and a dielectric material such as mineral oil, silicone oil, and paraffin wax) was used for initial LCEC experiments (ca. 1972) due to its excellent properties for organic electrochemical reactions. For most published applications of carbon paste electrodes, the useful lifetime of the electrode surface can extend to many months. However, if extremes of potential are used ($\geq$ 1 V vs. Ag/AgCl) and/or if the mobile phase contains high concentrations of organic solvent, the electrode surface must be renewed more frequently. Carbon paste is rarely used now, since most users prefer the convenience of glassy carbon. However, it is still hard to match the performance of a well-packed carbon paste electrode.

Glassy carbon falls more closely in line with our desire for a universal electrode material. Glassy carbon is a hard, amorphous carbon material capable of being polished to a mirror-like finish. When housed in a PEEK block, a glassy carbon cell offers good solvent resistance, a feature particularly useful with mobile phases containing acetonitrile or large percentages of methanol. It has been used in entirely nonaqueous systems with good success.

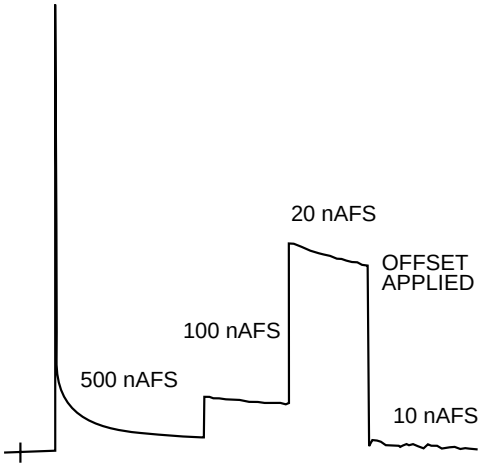
It is widely believed that sample molecules, lipids, or proteins will "poison" the electrode surface after a few samples have been injected. This notion undoubtedly arises from classical voltammetry (including polarography), where the presence of molecules of high molecular weight leads to serious problems. Voltammetric measurements quite often involve sample concentrations over the range 10^{-5} to 10^{-3} moles/liter. In some cases, the electrode reaction itself leads to polymer formation, which "passivates" the surface, causing unreliable results.

In the case of amperometric detection, the column isolates the electrode from many contaminants, the mobile phase continuously cleanses the surface, and the usual sample concentrations are in the range of 10^{-8} to 10^{-6} moles/liter. Furthermore, the electrode is only exposed to individual compounds for a minute or less due to the narrow elution zones encountered in liquid chromatography. All of these factors heavily favor the LC electrode in comparison with direct (i.e., no chromatography) electrochemical measurements. Hence, with LCEC experiments, electrode "life" is extended much longer than with traditional voltammetric experiments.

The LC-4C and LC-3C detectors set the reference electrode at ground potential and the working potential in reference to this. Other detectors (BAS 200B, LC-4B) may set either the auxiliary or the working electrode to ground potential. This situation often baffles those who have not been introduced to the simple fact that it is the POTENTIAL DIFFERENCE BETWEEN AN ELECTRODE AND THE SOLUTION which is of importance in electrochemistry and NOT the potential of the electrode material itself. Normally, this potential difference extends across an interphase region (the "electrical double layer") which is quite thin. It is the purpose of the electronics to control this potential difference while at the same time converting the current (resulting from the electrode reaction) into a voltage which is easily recorded or processed by a computer.

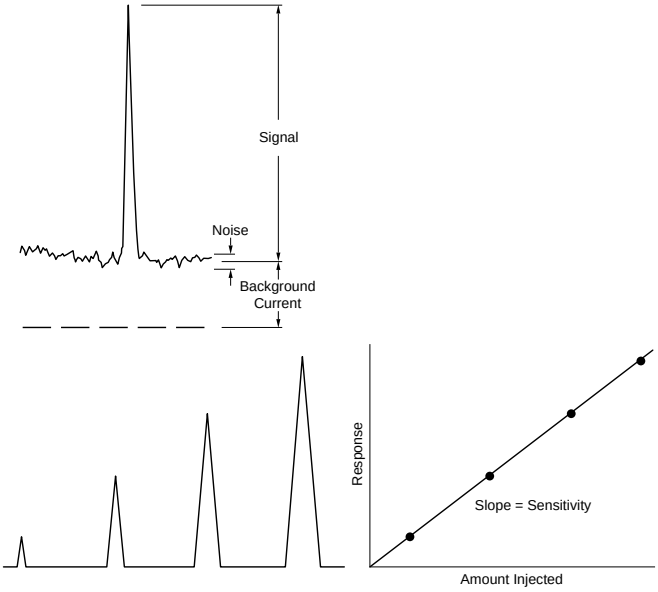
Figure 1.4 illustrates a recorder tracing encountered following initial application of a predetermined potential difference to a thin-layer cell containing a freshly prepared carbon paste electrode. The mobile phase is passing though the detector cell, but no sample has been injected into the chromatograph. Why then is so much current measured? Initially, some charge is required for the electrode/solution interface to achieve the applied potential difference. Additional charge will pass as the oxidation states of functional groups on the graphite are altered to a new state of equilibrium. Finally, impurities in the mobile phase and the mobile phase itself will oxidize (or reduce) resulting in a steady-state background current. Water will oxidize (or reduce) at all potentials although the rate of the reaction is quite slow except at very positive (or negative) extremes.

Figure 1.4. Typical equilibration response of electrode when it is first turned on.



The background current is electronically canceled by the offset current applied by the user. This results in a steady baseline at recorder zero (Figure 1.4). It is always desirable to operate with the minimum absolute background current to optimize sensitivity. The chromatogram is recorded "on top" of a current which often exceeds the peak heights for eluted compounds. High background currents increase the susceptibility of the instrument to noise (e.g., pump pulsations) and can result in serious nonlinear (negative) deviations in calibration curves.

Figure 1.5. Performance parameters for detector evaluation.



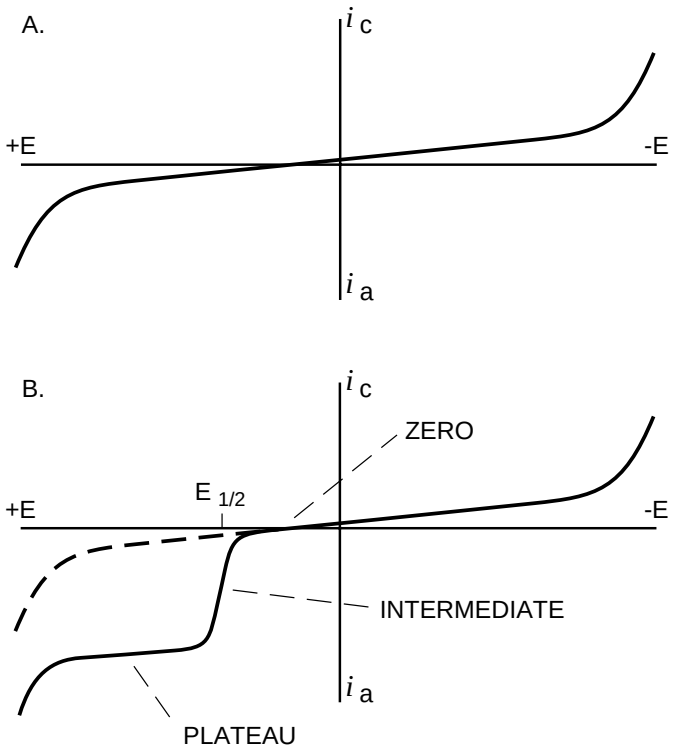
It is useful to evaluate the peak-to-peak baseline noise over a time period about ten times the width of the chromatographic peak (Figure 1.5). The frequency content of the noise relative to frequencies much higher or lower can usually be removed. The high frequencies are easily filtered out and the very low frequencies are, in effect, baseline drift which is eliminated when the peak is quantitated. The amount injected that would give a peak height equal to the background current noise is clearly below the limit of usefulness. An amount that would give a peak height five times the background current noise is useful under many circumstances.

Figure 1.5 summarizes in graphic form the various parameters useful in evaluating detector performance. These are often confused in real practice. Sensitivity is the slope of the response versus amount. Detection limits refer to the amount of analyte required to give a signal X times greater than the noise (usually X = 3). The two terms are not synonymous. The reader should be able to foresee situations in which two detectors demonstrate equal sensitivity but unequal detection limits. The only difference necessary is the noise. We recommend that LCEC users report sensitivity for each peak of interest as well as the noise. With this information, it is possible for the reader to predict a reasonable detection limit for each substance being determined.

1.2 Hydrodynamic Voltammograms

Let's begin our discussion by using a hypothetical electrochemical detector. Suppose we are operating the unit in an oxidative mode with a carbon electrode, and have initially chosen a potential low enough so that no detection of our test analyte should be possible. An injection of the test analyte is made; as predicted, no response is seen at the expected time. The potential is raised another 100 mV, the injection repeated, and the current response noted. This process is repeated until the potential becomes so high that the background current is prohibitive (usually +1.2 V). Suppose our test analyte were electroactive in the range examined. Then a plot of peak height versus applied potential would look similar to the solid line in Figure 1.6B. Three zones along this curve would become apparent.

Figure 1.6. Hydrodynamic voltammogram for mobile phase alone (A) and solute in mobile phase (B). The waveform for the solute is characterized by the half-wave potential $E_{1/2}$.



1. Zero current region. The potential was not sufficient to force oxidation to occur.

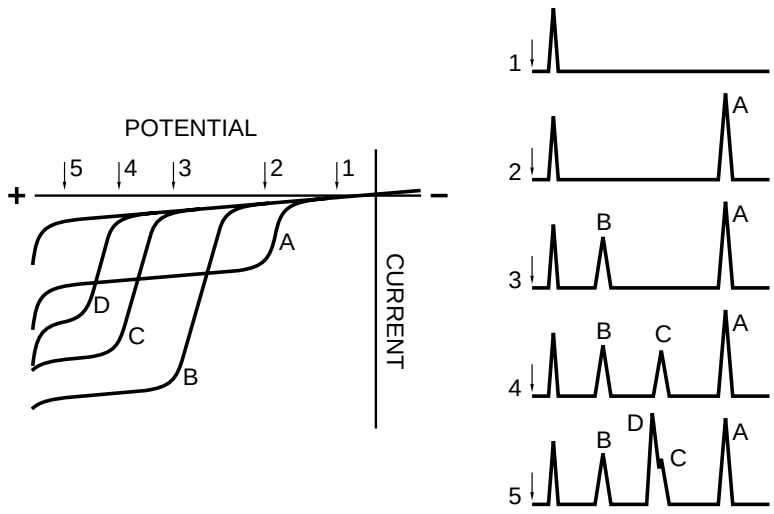
2. Intermediate region. The peak height is rising with increasing potential. Here the potential is controlling the kinetics of the heterogeneous electron transfer from the solute to the electrode surface.

3. Plateau region. In this zone, the peak height is independent of the potential. Diffusion to the electrode surface is the rate-determining factor; that is, the current is proportional to the rate of transport of molecules per unit surface area and per unit time.

In most situations, it is advantageous to operate the detector at a potential in the plateau region within 50–100 mV of the final break in the curve. Such a choice will offer maximum selectivity. The background current will be minimal and fewer interferences are likely. These advantages diminish when high potentials (beyond ±1 V) are required. This point is illustrated in Figure 1.7 where the voltammograms are shown for equimolar solutions of four oxidizable compounds (A–D). The curves have different oxidation potentials, as exemplified by the wave locations. Each compound also has its own particular limiting current, depending on such factors as the number of electrons transferred, the rate of that transfer from bulk solution to the surface, and the type of electrode surface used. Substance A, for example, is easier to oxidize than C, but yields a greater current response due to some combination of the above factors. Detecting A is very simple via LCEC. By setting the potential at 2, A may be clearly measured with no response from B, C, or D. In order to detect C, however, the potential must be set at a value which is sufficient for oxidation of B and A as well. Selectivity in this case is also dependent on the chromatography. Figure 1.7 also illustrates the chromatograms that would be obtained for potentials 1 through 5. Note that once in a plateau region, the current response for that compound is the same for any higher potential. Also, the farther one moves out in potential, the greater the number of compounds that will show up on a complex chromatogram. At potential 5, the selectivity (both electrochemically and chromatographically) is inadequate to permit separation of C and D.

Dual-electrode LCEC can overcome some of the limitations described above. Since BAS dual electrodes are interchangeable between parallel and series arrangements, and since different electrode materials can be used together in one cell, several alternatives not available in single-electrode cells become possible. In the previous example, it might be possible to distinguish between C and D based on a difference measurement in a dual series configuration. Detailed examples of the use of dual electrodes for complex problems are available in BAS application capsules.

Figure 1.7. Left: Hypothetical hydrodynamic voltammograms for four oxidizable solutes A, B, C, and D. Right: Hypothetical chromatograms for EC detector operated at potentials 1–5.

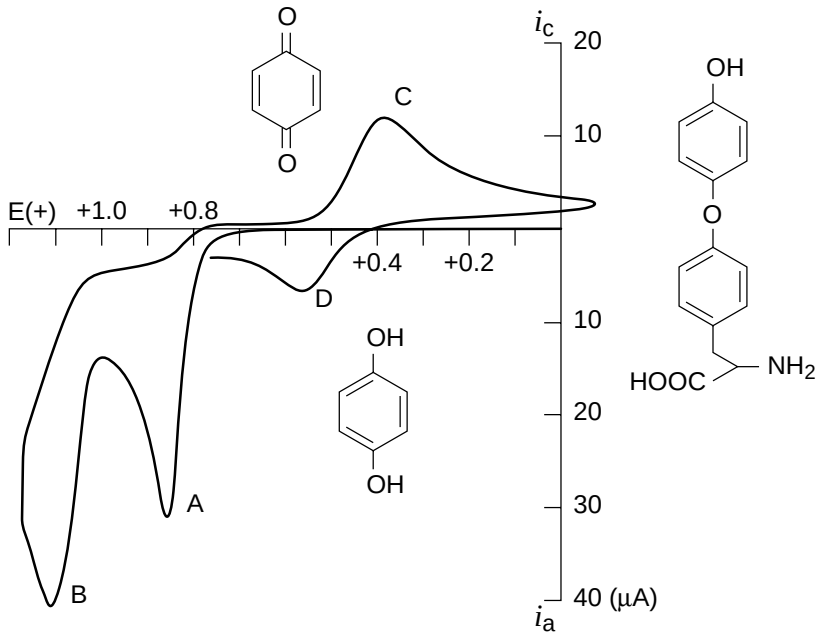


1.3 Oxidation or Reduction?

Most of the discussion thus far assumes that you have some idea of whether you will be looking at either an oxidative or a reductive electrochemical reaction. What if you don't know? Don't panic! It is not always obvious. Hydrodynamic voltammograms can give you some information, but they require several injections onto your LCEC analyzer, use mobile phase, and put wear on the column. There is an easier way.

Cyclic voltammetry (CV) is a quick and inexpensive means of learning the electrochemical behavior of the analyte in the mobile phase you plan to use. Mobile phase changes can cause shifts in applied potential. To keep the assay optimized, you should either do an HDV as described, or run a cyclic voltammogram on the sample in your new mobile phase. A sample cyclic voltammogram is illustrated in Figure 1.8. Section 8 lists CV data for a wide range of compounds. If you are continually faced with new analytical problems, you will find a CV instrument an invaluable accessory for LCEC.

Figure 1.8. This cyclic voltammogram of thyronine tells us that a potential of +0.85–0.90 V is necessary to detect it. It also undergoes an interesting follow-up reaction which could be detected by dual-electrode LCEC.



1.4 Reference Electrodes

The role of the reference electrode in any electrochemical cell is to provide a stable half-cell potential. Recall the Nernst equation, which states that the half-cell potential, E, is a function of the thermodynamic E°, measured at unit activity for all species involved, and the various activities of the components of the half-cell reaction.

$$E = E° + \frac{RT}{nF} \log K$$

K is the equilibrium constant for the half-cell reaction, written as an oxidation.

For the Ag/AgCl reference electrode commonly used in LCEC detection, the reaction and corresponding Nernst equation are as follows:

$$Ag° + Cl^- \rightarrow \underline{AgCl} + e^-$$

$$E = E° + 0.059 \log \frac{1}{[Cl^-]}$$

The activities of the Ag wire and AgCl are unity and, therefore, to a good first approximation, only the chloride ion concentration determines the E value.

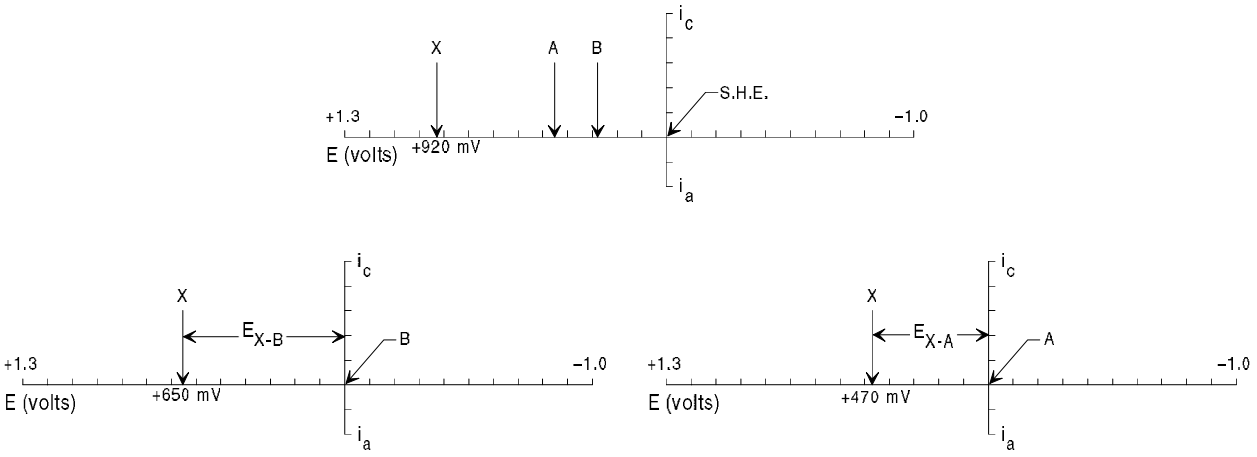
What affects the stability of the E value? From our experience, any minor drift in reference electrode E is usually due to a change in Cl^-. The concentration inside BAS reference electrodes is initially 3 M. Since the chloride ion concentration in the mobile phase is typically zero, a steep concentration gradient exists along the porous frit (at the tip) separating these two solutions. Since the concentration gradient allows the chloride ion concentration to decrease by continuous diffusion into the mobile phase, the potential must necessarily change as dictated by the Nernst equation. Although other chloride concentrations could be used with a reference electrode half cell, the problem of the concentration gradient would still exist, and drift in the reference electrode potential would likewise occur.

On a time scale of several weeks, the potential of the reference electrode half cell may drift to values on the order of 40 to 50 mV from when the reference electrode was originally installed in the chromatograph. On a short-term basis, this type of drift is not objectionable, because the change per day would only amount to a few millivolts. Since the applied potential required to force the LCEC oxidation or reduction to occur is generally set at least 20 to 50 mV in excess of that required, the detector's response factors are maintained at an adequate level. In isolated cases, however, the reference electrode may experience catastrophic failure. This degree of failure is indicated by the magnitude of drift in the reference electrode's potential, and the error normally amounts to +0.5 to +1.0 V. Obviously the reference electrode is not providing a stable potential. Usually, the cause of such deterioration is mechanical, and the reference electrode will require replacement.

In terms of caring for reference electrodes, a few guidelines are important. First, when using a reference electrode, always keep its tip wet. When not in use, the electrode should be stored in 3 M sodium chloride to prevent the problem of chloride depletion mentioned earlier. To prevent clogging of the porous electrode tip, do not keep the reference electrode in strongly nonaqueous solutions for any period of time. In reversed-phase liquid chromatography, however, mobile phases containing nonaqueous solvents are not unusual and some trade-offs must be made. In our laboratories, it is common practice to use a given electrode over a period of two weeks. When the reference electrode potential has drifted to an objectionable value, it is replaced with a new electrode. The old electrode may be partially restored by soaking it in a solution of 3 M sodium chloride, using the recommended storage container.

What value does the reference electrode have in the electrochemical cell? The half-cell potential of this electrode serves as the reference point along the potential axis by which we judge the oxidizing or reducing power of the working electrode in the vicinity of the interfacial region between the working electrode and the electrolytic solution. Suppose we have two different reference electrodes (Figure 1.9).

Figure 1.9. Potential axes for two reference electrodes (A and B) vs. the standard hydrogen electrode (SHE) (top). Referencing all potentials relative to reference electrode B would translate the potential axis (bottom left). The applied potential for a hypothetical analyte X will now be less when expressed versus reference electrode B. If reference electrode A were used, even less applied potential would be necessary (bottom right). Both situations provide equivalent oxidizing power.



Reference electrode A is situated at a potential more positive than that of reference electrode B. These potentials are dictated by variables such as the basic half-cell reaction, the concentration (formally the activities) of the participants in the half cell, temperature, etc. The potential axis upon which we have placed these two reference electrode half cells is a variable scale of oxidizing or reducing power. As we go to more positive potentials, the oxidizing power of the electrochemical detector increases; conversely, at more negative potentials, the power of the electrode to serve as a reducing agent improves. Suppose we impress between the reference and the working electrodes a potential difference of +0.5 V. As an example, let's take the compound caffeic acid, which oxidizes at approximately +500 mV with respect to the Ag/AgCl reference electrode. If we were to select a reference electrode whose potential relative to Ag/AgCl were 200 mV more positive than that of the Ag/AgCl couple, the applied potential required on the electrochemical controller to achieve equivalent oxidizing power would only be +300 mV. It is important to realize that the electrochemical potential axis is arbitrary in the sense that the reference electrode sets the zero point for this axis. For this reason, the reporting of electrochemical detector potentials must be referenced against the type of reference electrode used to complete the electrochemical cell. For example, it is commonplace to say caffeic acid oxidized at a potential of +500 mV vs. Ag/AgCl.

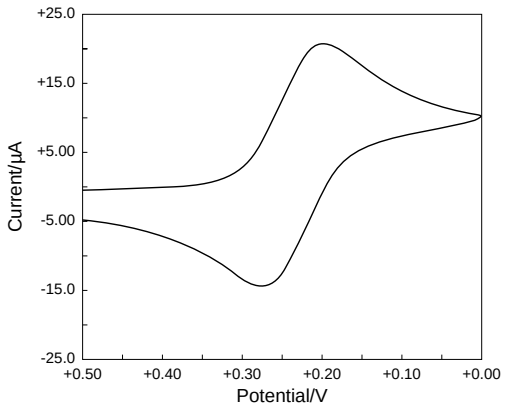
In the case of catastrophic failure, where the reference electrode potential might shift to a value of +500 mV with respect to its proper value, the performance of the electrochemical detector can be catastrophic as well. The electronic controller is still applying the same potential between the reference and working electrodes; it does not sense that a change in the reference electrode potential has taken place. Therefore, due to the shift, the actual oxidizing power of the working electrode has been increased to a sufficiently high level that objectionable and excessive oxidation of the mobile phase may occur (i.e., very large background current). You may not immediately discern whether the problem is due to the oxidation of some impurity in the mobile phase, deterioration of the working electrode surface, or a catastrophic change in the half-cell potential of the reference electrode couple. The problem must be diagnosed using a trial and error procedure where each of the above variables is tested independently of the others.

Since the half-cell potential of the reference electrode is taken as the arbitrary zero point along the electrochemical potential axis, it should not be surprising that oxidations may take place at negative potentials and reductions may take place at positive potentials. The positive or negative sign is merely an indication of where, in terms of absolute oxidizing or reducing power, the working electrode sits relative to the reference electrode. For example, if we selected a reference electrode whose potential was +1.0 V with respect to the present Ag/AgCl reference electrode potential, then nearly all electrochemical oxidations and reductions would be reported at negative potentials versus this new reference electrode. Specifically, in the case of pentachlorophenol whose oxidation potential vs. Ag/AgCl is approximately +800 mV, the new oxidation potential for an equivalent response would now be –200 mV because we would need to lower the working electrode's potential by that amount to make its oxidizing power equivalent to that when Ag/AgCl was used. For the reduction of a compound such as nitroglycerin where the applied potential vs. Ag/AgCl was –1.0 V, the new potential would be –2.0 V versus the new reference electrode.

Thus, we have transformed all of our oxidation and reduction potentials to a series of electrochemical potentials that are all negative, even though some of these are oxidation reactions where electrons will be passed into the electrode surface. Although it may seem contradictory that electrons would flow into an electrode with a negative potential, the issue here is not the charge on the electrode surface, but the establishment of a gradient of potential at the electrode/solution interface that is sufficient to cause the oxidation or reduction to occur. This *difference in potential across the interface* is what concerns the electrochemist! In all the cases we have described, the same interfacial potential difference would exist. A few more examples may be in order.

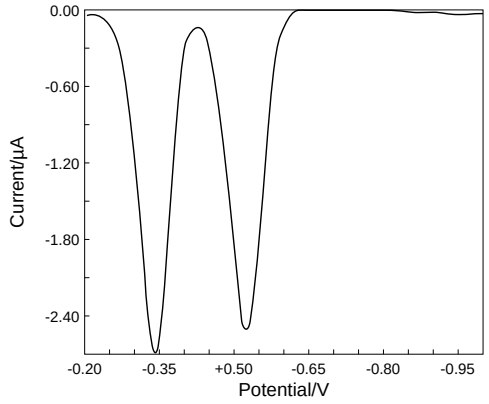
Let's take the case of the well-behaved electrochemical couple ferricyanide/ferrocyanide. This is a one-electron oxidation between two forms of iron held in a very stable hexacyano complex. Figure 1.10 demonstrates the oxidation and reduction of these complexes by cyclic voltammetry. Both reactions occur at positive applied potential.

Figure 1.10. Oxidation and reduction of iron hexacyano complex using cyclic voltammetry.



Another application familiar to all electroanalytical chemists is anodic stripping voltammetry. In this technique, the potential of the working electrode—usually mercury or mercury-coated graphite—is held at a sufficiently negative potential, typically –1.0 V vs. Ag/AgCl, while trace metals are deposited to form a concentrated amalgam of metallic atoms in the zero oxidation state. After a sufficient deposition period, the potential is scanned in the positive direction, and the metal atoms are characteristically stripped from the mercury into solution via a forced oxidation to their ionic states. The potential of each of these stripping reactions occurs at a value characteristic of the metal ion being analyzed (Figure 1.11). Note that all of these oxidation reactions are done at negative potentials versus the reference electrode implied.

Figure 1.11. Anodic stripping voltammetry of trace metal ions using a BAS 100B/W Electrochemical Workstation and an Hg/Au voltammetry electrode. The stripping peaks correspond to the oxidation (all at negative potentials) of trace metals electrodeposited in the mercury.



In summary, we should make clear the following points about reference electrodes and their use:

1. The reference electrode serves as our electrochemical zero point on the applied potential axis, and for this reason, when potentials are reported either for cylic voltammetry, LCEC, or other purposes, the reference electrode must be specified.

2. The potential of the reference electrode is determined by the half-cell couple involved. In the case of Ag/AgCl, the reference electrode potential is determined by the integrity of the Ag/AgCl wire in the electrode and the concentration of chloride ion in the reference electrode filling solution. The concentration of chloride ion in the reference electrode will necessarily change with time due to the concentration gradient across the reference electrode frit. This small change in the reference electrode potential may be retarded by careful storage of the reference electrode in 3 M sodium chloride. Alternately, the change may be compenated by a corresponding change in the potential applied to the cell by the electrochemical controller.

3. The sign of the applied potential is determined by the relative magnitude of the oxidizing or reducing power desired, relative to the reference electrode's potential. There are several types of reductions that may be carried out at positive potentials and an equally diverse number of oxidations that may be done at negative potentials with respect to the Ag/AgCl couple. Keep in mind that these sign conventions are relative and not fixed.

1.5 Mobile Phase Limitations

Since amperometric detection depends on the transfer of electrons between a solute and the electrode surface, it is important to choose a solvent (mobile phase) that effectively permits the electrode reaction to occur. The primary limitations on the mobile phase are:

1. ELECTROLYTE MUST BE PRESENT, usually at 0.01 M to 0.1 M concentrations, to convey charge through the electrochemical cell.

2. The solvent must have a sufficiently high dielectric constant to freely permit IONIZATION OF THE ELECTROLYTE.

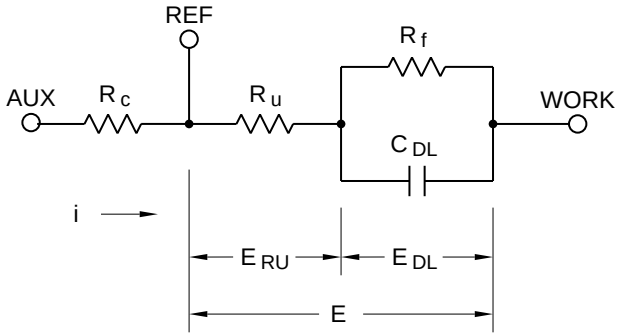
3. The mobile phase (electrolyte + solvent) must be ELECTROCHEMICALLY INERT at the electrode surface; that is, the background current at the applied potential should be negligible, with no chemical deterioration of the surface.

Even under the above restrictions, the scope of LCEC is very wide, since all ion-exchange and most reversed-phase separations employ these types of mobile phases. Initially all reports on LCEC dealt exclusively with ion-exchange columns using aqueous buffers. A majority of the separations performed with LCEC are now done with reversed-phase packing materials (e.g., BAS Biophase ODS or Phase II bonded phases). By bonding silyl hydrocarbon chains to a particle of silica, one fashions a packing capable of retaining nonpolar and weakly polar solutes from polar mobile phases. The versatility of reversed-phase materials can be further extended to ionic solutes by adding small amounts of ion pairing reagents to the mobile phase (e.g., BAS P/N CF-1090 sodium octyl sulfate). Polar bonded phases (diol or nitrile) have also been used. Few applications with silica gel columns have been reported, since the mobile phases needed to effect separations on these materials (nonpolar solvents with low dielectric constants) are incompatible with the requirements of LCEC. There are some ways to get around this problem with nonaqueous solvents.

1.6 Uncompensated Resistance

The simplest electronic equivalent of a three-electrode electrochemical cell would resemble Figure 1.12. The goal of the instrumentation is to impress all of the potential applied by the potentiostat across the interface between the electrode surface and the solution. This presents the sharpest potential gradient to the molecules.

Figure 1.12. Simple electronic equivalent of a detector cell.



Since the potential is applied between the reference and working electrodes, the user's control of the distribution of this potential between R_u and the interfacial double-layer impedance is impossible. The reference electrode may be moved closer to the working electrode (thereby minimizing R_u), but the term R_u will always be present to some degree. For this reason, R_u is termed the uncompensated resistance. C_{DL} is the double-layer capacitance (this capacitance is charged up when the detector is turned on, and is the cause of the initial off-scale transient) while R_f is the faradaic resistance. The latter term represents the resistance to charge transfer across the interfacial region.

Since uncompensated resistance is always present, the simplest expression possible for the applied potential, E, is

$$E = E_{RU} + E_{DL}$$

Figure 1.12 describes this distribution. The difference between E and real applied potential E_{DL} is the term E_{RU}. Since the electrical equivalent in this region is a simple resistor,

$$E_{RU} = iR_u$$

where i is the current passing through R_u (and the double layer) at any time. This factor, dependent on both the current and the resistance in the cell, is referred to as "iR drop."

The cells described in Figures 1.2 and 1.3 possess very low uncompensated resistance. The electrodes face each other directly across the thin-layer channel. Hence, even if cell currents reach hundreds of microamperes, E_{RU} remains negligible and the interfacial potential remains constant.

The "curse" of high uncompensated resistance is narrow dynamic range. Spatial relationships within electrochemical cells are critical, and the compact nature of these two updated thin-layer designs is optimal in this regard.

1.7 The Current Response

Two principal contributions to the current response of a thin-layer electrochemical cell are encountered. These are:

1. The FARADAIC RESPONSE, due to redox processes either from analyte or solvent impurities, and

2. The CHARGING CURRENT, required to charge the double-layer capacitance at the solution/electrode interface.

Normally, the charging current is not an issue since the detector is operated at fixed potential. Once the potential is supplied to the cell, and the background decays to an acceptably flat level, only faradaic contributions exist.

The fundamental relation for LCEC operation,

$$i = nFAJ_{x=0}$$

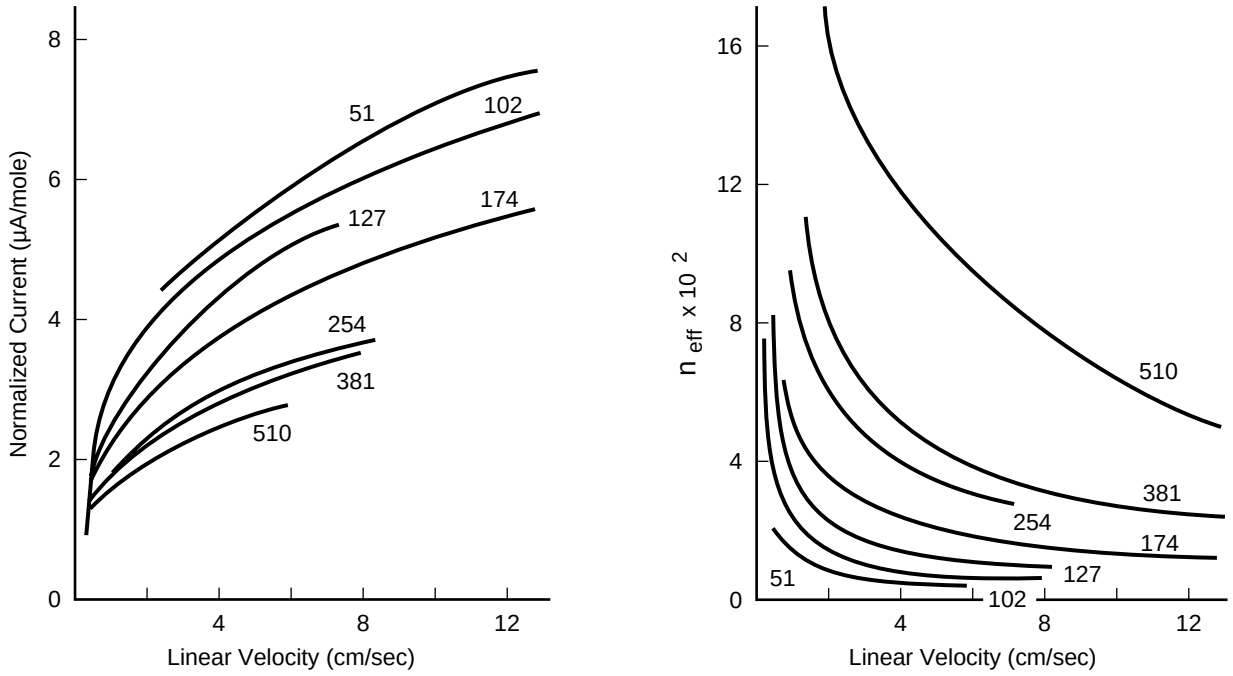
describes the current in terms of the flux J and various constants. The flux is dependent on the flow rate, cell dimensions, and the diffusion coefficient and concentration of the analyte being oxidized or reduced. A rigorous derivation incorporating a detailed expression for the flux permits the following equation:

$$i_{lim} = 1.467\ nFAC_o\ (D/h^{2/3})\ (U_V/d)^{1/3}$$

where n is the number of electron equivalents/mole, F is Faraday's constant, A is the area of the electrode (cm^2), C_o is the concentration of reactant in the bulk eluent (mol/cm^3), D is the diffusion coefficient (cm^2/s), h is the thickness of the channel, U is the volume flow rate (cm^3/s), and d is the width of the channel. From this expression, it is evident that the diffusion-limited current should be proportional to the concentration of the analyte, the area of the electrode, and the cube root of the velocity through the cell, and inversely related to the cube root of the channel thickness.

Figure 1.13 illustrates the plot of the current response versus linear velocity (cm/s) through the cell for a number of channel thicknesses. The current response was normalized by dividing the peak current by the concentration of the injected analytes. Faster flow rates favored greater peak heights. A similar plot was made of the normalized coulometric (area) response versus the linear velocity. The coulometric response decreased with increasing velocities. Remember that the current response describes the rate of conversion, while the coulometric response relates to the amount converted. The rate can be high in spite of low amounts converted and vice versa.

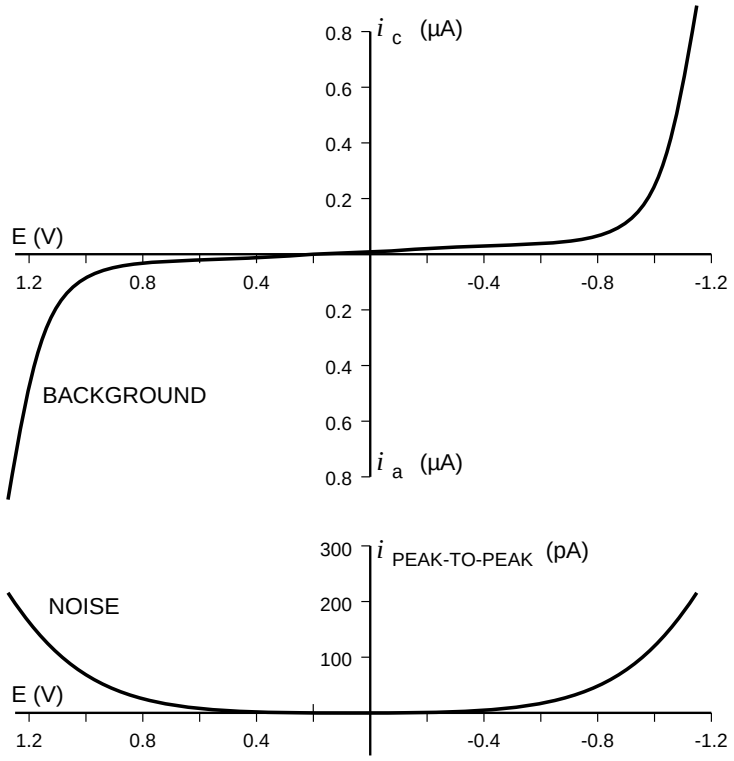
Figure 1.13. Peak height (left) and peak area (right) versus linear velocity for various channel thicknesses (indicated in μm).



1.8 The Background Current

The background current is principally faradaic current arising from the oxidation (or reduction) of electroactive impurities in the mobile phase. It is entirely analogous to the background absorbance in ultraviolet detector systems. Common sources of background current are oxidation or reduction of the mobile phase solvent or buffer salts, oxygen (reductive), ferrous iron (oxidative), metal ions (reductive), etc. For the simple and usual case where the background current is caused mainly by the mobile phase, a plot of background current versus applied potential would resemble Figure 1.14. Note that the current is exponentially related to the applied potential at both a positive and a negative limit. Within these limits, however, a fairly flat low background response is typical.

Figure 1.14. Generally, the greater the background, the greater the noise. Both follow the same trend.



1.9 Noise

Noise is the random or periodic pattern superimposed on the steady-state background signal. Usually measured from peak to peak, the noise represents the summation of spurious contributions from pump pulsation, flowcell hydrodynamics, surface reactions, static electricity, power-line noise, and electronic amplification. Obviously it is desirable to minimize noise. Specific guidelines are given below; detailed advice is outlined in Section 6.

1. Select a pump capable of delivering pulseless flow relative to the detection limits you desire. Use pulse dampers. Use a complete BAS LCEC Analyzer system.

2. Passivate your liquid chromatograph frequently. Check with the manufacturer for recommendations on this. Usually, an acid washing (e.g., 6 N HNO_3) is employed. Be sure to disconnect your column and cell before attempting this procedure (see Section 7).

3. Follow good laboratory practice and maintain the pump seals and check valves in top working order. Lubricate the pump or maintain as specified by the manufacturer. Be fastidious about flushing the system thoroughly (with a mobile phase devoid of salts, e.g., 40% CH_3CN:H_2O) when you don't plan to use it for a long period. This helps eliminate random flow fluctuations.

4. Connect all components in your system to the same electric circuit to avoid ground loops.

5. Avoid solvents capable of destroying the electrode surface. This is of concern primarily for carbon paste or chemically modified electrode surfaces.

How do the background current and noise affect performance? As with most other quantitative measurements, the noise with an electrochemical detector is dependent on the magnitude of the background signal. Generally, the greater the background, the greater the noise, and the ratio of noise to background current stays approximately the same. This trend is true for the amperometric detector and glassy carbon materials. The noise will follow the same trend as the background current (Figure 1.14). It is impossible to operate at extremely high gain with high background currents if trace measurements are to be achieved.

1.10 Attaining Good Signal-to-Noise Ratios

The parameter most useful for analytical comparison is the signal-to-noise ratio (SNR or S/N). An extremely responsive electrode may be equally noisy, just as the apparent baseline quiescence of another electrode may be due to passivation, thus rendering it worthless. Neither situation may be evaluated by comparing just the signal (the response from injection of analyte) or just the noise. For this reason, the SNR is most pertinent.

Response should be directly proportional to surface area. Therefore, if we were to use an electrode of 10 times the area, it should give 10 times the response. Such improvement would be highly significant, if the cell noise were to remain at its original value. However, if the noise were to increase proportionately, the use of a larger surface would be questionable.

In actual practice, larger surface area electrodes are less useful. Figures 1.15 and 1.16 illustrate this trend. Both the analyte signal (peak height) and peak-to-peak noise are plotted. Each increment of added area adds proportionately less to the output signal, but adds linearly to the output noise. The smaller the electrode, the greater the signal-to-noise ratio. For this reason, large plate electrodes, porous flow-through cells, and other large surface electrodes demonstrate no advantages in terms of the SNR. If you intend to use the "Jumper" to couple the dual electrodes together, you should expect to see a less favorable SNR. The experiment might still be useful if you are trying to differentiate a particularly small peak and need a larger signal. Since noise depends on potential, it follows that the smallest minimum detectable quantities will occur in cases in which the substances of interest are easily oxidized or reduced. Figure 1.17 demonstrates the value of judicious potential selection.

Figure 1.15. Signal and noise for electrodes of different surface areas. The solid lines indicate the increase in current expected for a linear response in area.

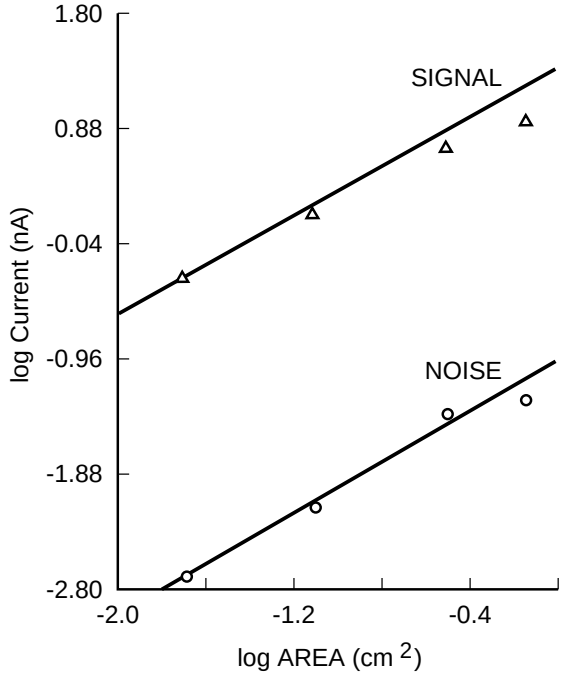


Figure 1.16. As the electrode surface area increases, the signal-to-noise ratio becomes unfavorable.

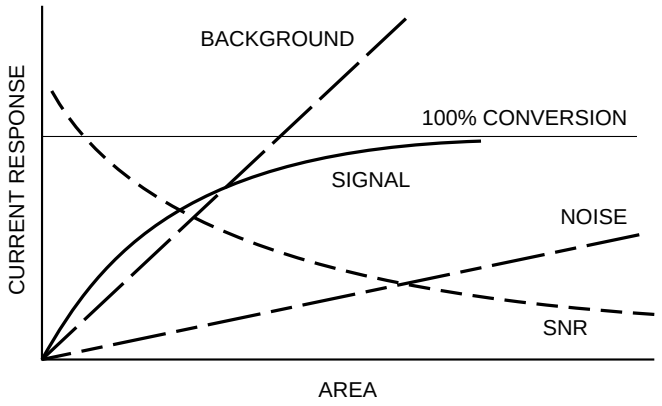
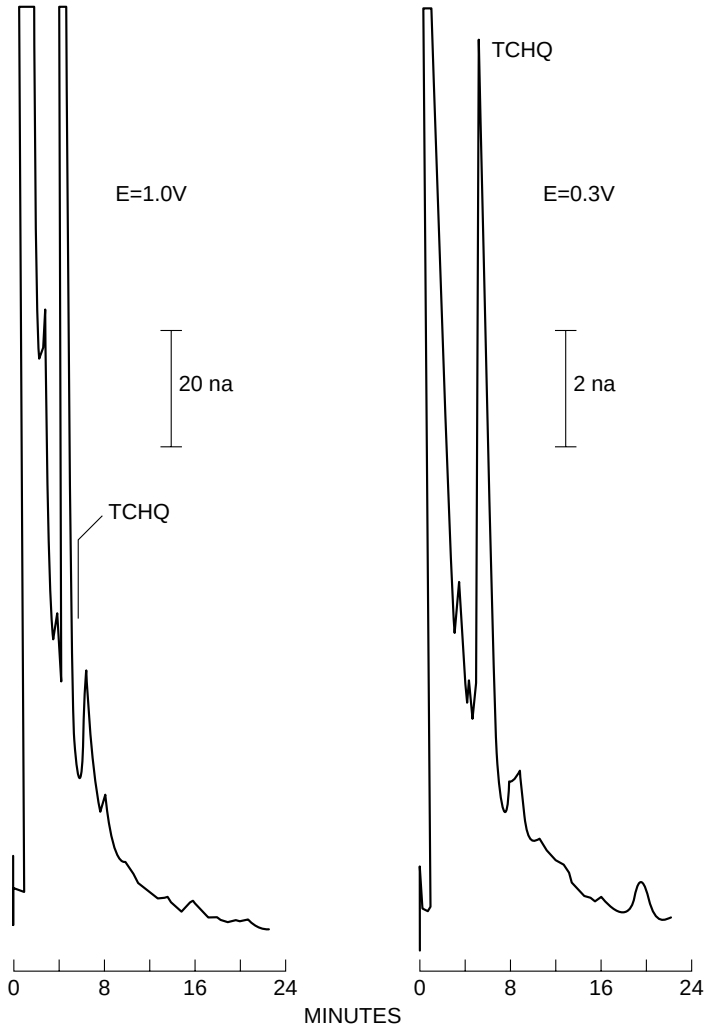


Figure 1.17. Detection of tetrachlorohydroquinone (TCHQ) at two different applied potentials. This illustrates the specificity of electrochemical detection, which can in turn influence the sensitivity. Determination of the optimum applied potential is not unlike determination of the optimum wavelength used in UV detection methods. See Sections 1.2 and 1.3.



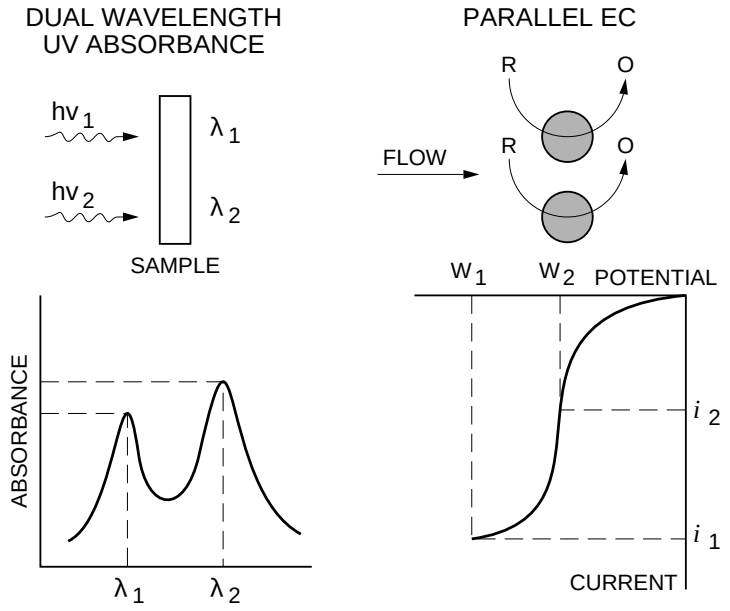
1.11 Dual Mode LCEC

The electrochemistry for dual-electrode LCEC is governed by the same variables described previously. With this instrumentation, the electrochemistry can be manipulated to improve sensitivity and extend the applications of electrochemical detection.

To understand these options, the following discussion will make reference to common spectroscopic techniques that are familiar to the reader. Analogies can be made on a number of points. In spectroscopy, the response of a compound to light energy is recorded as an absorbance spectrum, or in the case of fluorescence, as an excitation/emission spectrum. In electrochemistry, the current response is plotted against the applied potential. When the potential scan is reversed and plotted, the electrochemical equivalent to a fluorometric emission spectrum is obtained.

How does this apply to dual-electrode LCEC? The analogies are shown in Figures 1.18 and 1.19. In the DUAL-PARALLEL (adjacent) configuration, the detection options available are similar to those available when using a dual-wavelength photometric detector. The eluent from the LC can be monitored at two independent applied potentials. A simultaneous profile of reducible and oxidizable analytes can be obtained. Also, response ratios can be calculated to provide qualitative information on a particular chromatographic peak. Going back to our spectrophotometric analogue, the current ratio i_1/i_2 is conceptually the same as the absorbance ratio A_1/A_2. Both are constants which can be used for peak identification.

Figure 1.18. An analogy can be made between DUAL-PARALLEL amperometric detection and spectroscopic measurements. Enhanced selectivity is often the end result.



Simultaneous measurement in the DUAL-PARALLEL amperometric detection mode can be used to determine more than one compound in a chromatogram. For example, the detector potential at one electrode may be set sufficiently positive to oxidize all compounds of interest and the second electrode may be set at a substantially lower oxidizing potential to only react with those compounds that are electrochemically active at these lower potentials. Thus, the resultant dual tracing will allow quantitation at both applied potentials, and the lower-potential chromatogram will be more discriminating, improving the selectivity of the measurement.

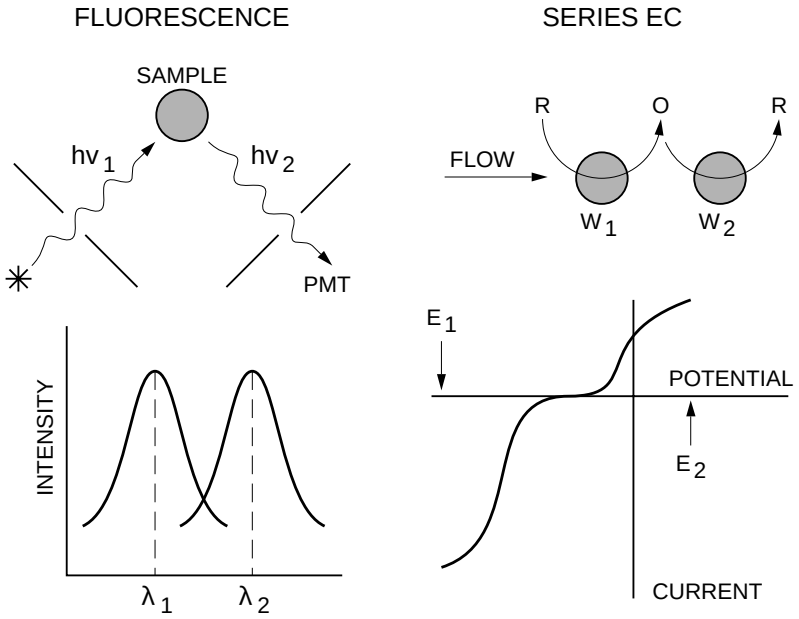
Another application of the DUAL-PARALLEL mode is to measure the difference chromatogram. This would be similar to choosing a particular potential window to monitor. In this mode, the working electrodes are poised in a potential region where the response of the analyte of interest is dramatically different; that is, where the slope of the current-potential curve is large. The difference chromatogram enhances specificity by only displaying those compounds that are electrochemically active in the region between the two set potentials; all other responses, whether noise or analyte signal, are subtracted out. Of course, one electrode can be measuring an oxidation while the second can be monitoring a reductive reaction.

The DUAL-SERIES electrode configuration most closely resembles fluorometry. This analogy is shown in Figure 1.19. As in fluorescence, a reactive "intermediate" is produced by some excitation function which yields a product that generates some measurable response. In the case of fluorescence, it is the emission signal; in the case of the electrochemical detector, it is the redox couple's follow-up reduction (oxidation) reaction. In fluorescence, "quantum efficiency" is the measure of the maximum amount of fluorescence available from a given input intensity. A similar term in series-dual electrochemical detection is "collection efficiency," the ratio of the current at the downstream electrode to the current at the upstream electrode:

$$\text{collection efficiency} = \frac{\text{current}_{\text{downstream}}}{\text{current}_{\text{upstream}}}$$

As quantum efficiency is a statement of the expected response for a given compound under a specified set of conditions, so is collection efficiency. A range of collection efficiencies can be obtained depending on cell dimensions (distance between electrodes and the ratio of lengths of the electrodes along the flow axis) and the homogeneous chemistry (hydrolysis, coupling, etc.) that might take place prior to arrival at the downstream electrode. The maximum range of values is 0.37 to 0.42 for reversible compounds at equal-surface-area planar electrodes in a standard cross-flow thin-layer cell at normal flow rates (1 mL/min). In a standard cross-flow thin-layer cell configured for microbore chromatography (16 μm thin-layer gasket and < 100 μL/min flow rate), a collection efficiency of > 0.85 is observed.

Figure 1.19. DUAL-SERIES amperometric detection methods can also enhance selectivity and thus improve detection limits. An analogy can be made to fluorescence measurements for applications that require a reaction at the upstream electrode to create a product detectable at the downstream electrode.



DUAL-SERIES electrochemical detection can, in certain cases, improve selectivity and detection limits. Compounds with higher collection efficiencies will dominate the response at the downstream electrode and can be measured with improved selectivity. Not all compounds have a reversible redox couple; these will not react at the downstream electrode. This selectivity may be advantageous. The upstream electrode functions as a derivatizing (GENERATOR) electrode, while the downstream electrode detects the product(s) created upstream. There are many applications of these scheme, but the major use is to improve the detection of compounds by generating electrochemical products that can be detected at a more favorable potential (where noise and interferences are less). This is successful because most background (media) reactions are chemically irreversible (e.g., reduction of oxygen in aqueous media, reduction of hydrogen ion, and oxidation of water).

When the same two SERIES electrodes are operated at the same potential, and the difference response is recorded, the result will be approximately 40% of the difference between the upstream and downstream electrode responses. Responses that would be equal at the same potentials, such as background currents and other factors contributing to noise, are nulled out.

You may find the following references more detailed and informative:

1. Roston, Shoup, and Kissinger: Anal. Chem. 54 (1982) 1417A.

2. Goto, Sakurai, and Ishii: J. Chromatogr. 238 (1982) 357.

3. MacCrehan and Durst: Anal. Chem. 53 (1981) 1700.

4. Roston and Kissinger: Anal. Chem. 54 (1982) 429.

5. Shoup and Mayer: Anal. Chem. 54 (1982) 1164.

6. Mayer and Shoup: J. Chromatogr. 255 (1983) 533.

7. Lunte and Kissinger: Anal. Chem. 57 (1985) 1541.

Section 2. Detector Electrodes

2.1 Electrodes as Active Surfaces

In electrochemical detection, the signal being monitored is a direct response to an ACTUAL CHEMICAL REACTION, as compared to the physical measurement occurring in other LC detectors (e.g., refractive index, absorbance, fluorescence). Electrochemical detectors sometimes get the reputation of being "finicky" compared to optical detectors, but it is important to realize that they behave in a very different manner, and this difference is responsible for the higher degree of sensitivity you can expect from EC methods. You will learn to handle electrochemical detection the same way you would handle any chemical reaction, by considering several variables which can influence the outcome of the reaction. In LCEC, the reaction product that concerns us is the current (i.e., the response, or signal).

The response is dependent on the chemical (electrochemical) reaction variables. These include the electrode surface where the reaction is taking place, the mobile phase (reaction medium), and the compound undergoing the reaction. The fundamental electrochemical relationships for this mode of detection were discussed in Section 1. This section will go into greater detail on some of the practical aspects of the electrodes, including electrode materials, detector cell design, solvent considerations, and the maintenance, service, and performance of each.

The general requirements for electrochemical detection are that the mobile phase be conducting, the working (detector) electrode be chemically inert, and the analyte be electrochemically oxidizable or reducible at the electrode surface in the chosen solution. Solution conductance is met by having an electrochemically inert salt (an ionic conductor) dissolved in the mobile phase. This places some restrictions on the mobile phase composition. Usually, aqueous or partially nonaqueous solutions are used, though a totally nonaqueous solution can be used as long as an appropriate salt is dissolved in it. Since the majority of the liquid chromatographic separations being performed today use reversed-phase packing materials, this requirement is easily met. It is also advisable that the mobile phase be a buffer solution for both electrochemical and chromatographic reasons.

Ideally, the working electrode should be inert to the electrolytic solution and only respond to the analyte in a thermodynamically defined, potential-dependent fashion. Many times this is not the case. The kinetics of heterogeneous charge transfer between the electrode and the analyte, in addition to the reactivity of the electrode itself, enter into the situation. For example, Figure 2.1 shows actual current-voltage curves (normalized hydrodynamic voltammograms; see Section 1 for further discussion of these waveforms) for the oxidation of a substituted *o*-hydroquinone on four carbon-based electrodes. The sharpest break with potential occurs on the CP-O while the broadest voltammogram occurs on the CP-W, indicating that faster electron-transfer kinetics occur on CP-O relative to CP-W. The CP-S and glassy carbon electrode materials exhibit kinetics similar to CP-O. Fast electron-transfer kinetics characterized by sharply rising voltammograms improve the selectivity of the overall determination. Some carbon paste formulations (graphite mixed with hydrocarbon and fluorocarbon polymers) show slower kinetics, similar to the CP-W described above. The

sensitivity or response for a given amount injected is approximately the same in the diffusion-limited region, but for these materials, a greater potential is usually required for equivalent response.

Electrochemical reactivity can be altered considerably by changing the electrode material. In many cases, this can be highly advantageous. The large hydrogen overpotential characteristic of mercury electrodes in protic solutions extends the attainable negative potential range (past carbon) and makes difficult reduction reactions possible. For this reason, mercury remains the material of choice in these potential regions. However, the reduction of dissolved oxygen, a very facile reaction on mercury over a wide potential range, does not occur until well into the negative-potential region on a glassy carbon electrode. Thus, the oxygen overpotential of glassy carbon is much better than that of mercury (Figure 2.2) and precludes the need for oxygen purging at moderately negative potentials.

Not all electrode materials will withstand solvents. All carbon-paste formulations are limited to varying degrees (usually not more than 10% organic solvent), depending on the graphite binder. Glassy (vitreous) carbon, platinum, and mercury (amalgamated gold) are far more resistant to organic solvents.

All electrode materials require some surface conditioning or modification before they stabilize to a constant background current level. The conditioning process is observed as a slowly decaying current output from the detector after the electrode is turned on. This may take only a few minutes for an electrode that has been switched off momentarily to as long as a few hours for a freshly prepared glassy carbon electrode at high negative or positive potentials. Longer stabilization times will be required for high-sensitivity operation.

Figure 2.1. Hydrodynamic voltammograms for various carbon pastes and glassy carbon working electrodes.

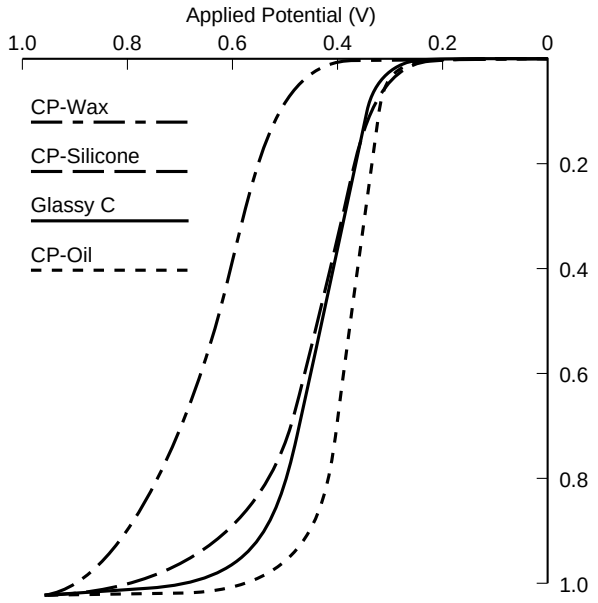
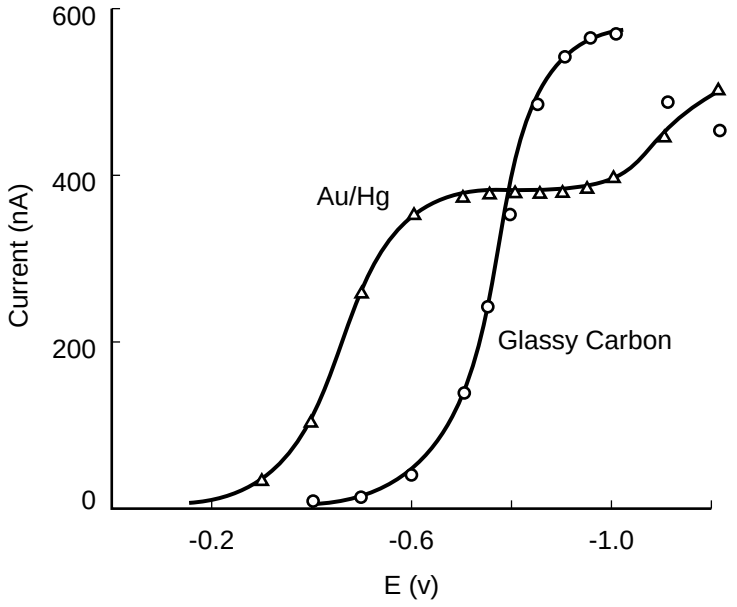


Figure 2.2. Hydrodynamic voltammogram for oxygen on glassy carbon and mercury electrodes.



2.2 UniJet Detector Cell

The UniJet detector is a new addition to the BAS line of amperometric detectors for liquid chromatography. The detector has been designed with microbore chromatography in mind. Due to the stringent requirements of minimal dead volume in microbore systems, the UniJet detector was designed as the end fitting of the SepStik microbore column. In order to keep the overall size and the internal volume to a minimum, a radial flow pattern was used (Figure 2.3; this is not wall-jet hydrodynamics). In comparison to the more traditional approach of a cross-flow cell, the radial flow cell gives improved response at microbore flow rates ($\leq$ 200 µL/min) and less dilution of the sample before the detector. Figure 2.4 illustrates the improved response for the UniJet cell compared to the classical BAS detector cell under microbore conditions. In addition to the above improvements, the radial flow profile allows for more rapid equilibration of the electrode. The UniJet detector cell does allow for a variety of reference electrodes used without a liquid junction (salt bridge).

Figure 2.3. Flow patterns for cross-flow and radial cells.

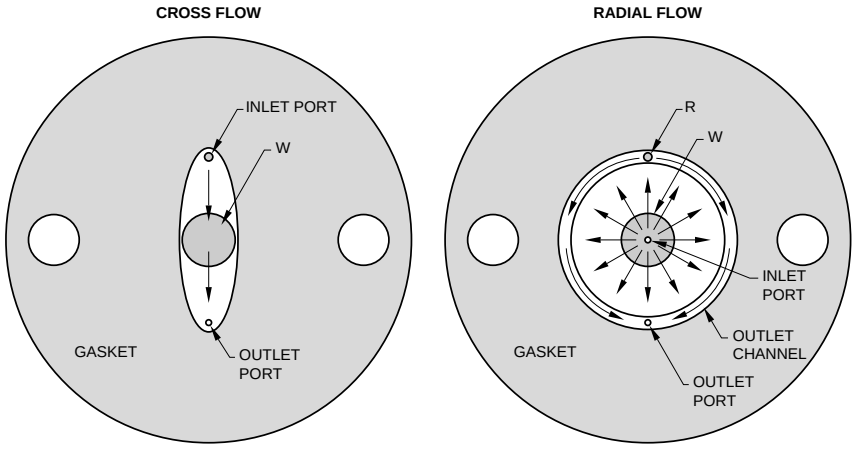
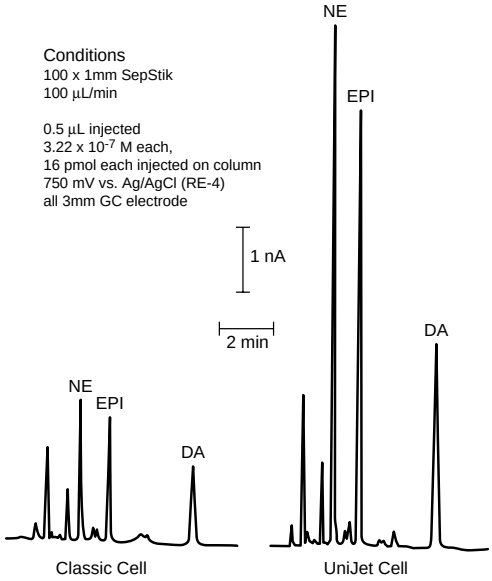


Figure 2.4. Comparison of chromatograms from BAS classic and UniJet cells at low flow rates.



2.3 Leaving the Electrode "ON"

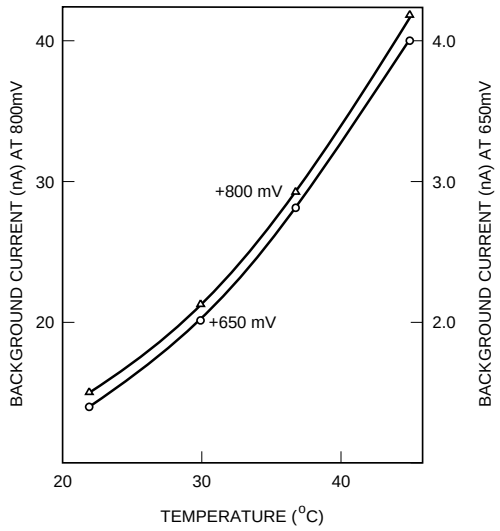
If the LC system is being used on a daily basis, the electrode can be left "ON" continuously. Make sure you have plenty of mobile phase in the reservoir, or route the outlet from the LCEC cell to the reservoir so you "recycle" the mobile phase until you return. The startup time the following morning is substantially reduced.

Section 3. Temperature-Stabilized Detector Operation

3.1 Rationale for Use

Thermostatted control of the eluent temperature before it reaches the cell reduces the effects of ambient temperature change on electrochemical detection. Furthermore, temperature control of the column protects the separation (peak retention and width) from the effects of temperature change, and when the LC-23C cartridge column heater is used with the cross-flow cell package, the distance between the heated column and cell inlet is quite small and there will be sufficient temperature control to minimize effects on detection. However, in extreme environments (e.g., lab temperature changes due to automated heating/cooling shutdown after business hours, or proximity to vents, ducts, or drafts) or at high detector gain, small fluctuations in temperature at the detector cell can still produce marked deviations in the detector baseline. In these cases, both a column heater and a cell preheater may be needed. Figure 3.1 shows plots of EC detector background current versus temperature. The slope of the curve (di/dT) is significant, typically 0.5–1.5 nA per °C. Hence it is easy to see how a small change in eluent temperature (e.g., 0.1 °C) could still cause appreciable shifts in the baseline.

Figure 3.1. Plot of electrochemical background current versus mobile phase temperature on a glassy carbon electrode operated at 650 and 800 mV applied potential.



What phenomena are responsible for this dependence? The background current in electrochemical detection derives from several contributions, the majority component being the oxidation or reduction of the solvent itself.

$$2H_2O \longrightarrow O_2 + 4H^+ + 4e^-$$

For water, the reaction is sluggish at moderate potentials; this is due to poor heterogeneous electron-transfer kinetics at the electrode surface. Elevations in temperature increase the heterogeneous rate constants, and the background current (the measure of the rate of electron transfer) correspondingly increases. From a noise standpoint, if we must operate at elevated temperature, we must do so precisely. In many cases, only a small rise over ambient is a good compromise. In doing so, one gains control of environmentally induced baseline drift without fighting large temperature coefficients.

Elevated temperatures similarly affect the magnitude of the peak current. It is not unusual to increase peak currents 50–70% (over ambient) by elevated-temperature operation. Although the temperature dependence of diffusion coefficients alone cannot explain this, it is probable that the diffusion layer thickness decreases as the viscosity drops. The concentration gradient at the electrode surface is accentuated, and the end effect is larger peak currents.

Taken separately, the trends in both background current and peak current versus temperature are inadequate in predicting the effect, if any, on detection limits. When the pertinent data are properly expressed in terms of the signal-to-noise ratio, the improvement is not so dramatic. For example, operation at 55 °C requires more vigorous temperature precision than at 35 °C. Thus, a 1.8-times increase in peak current is counterbalanced by a 23-times increase in baseline noise. A small increase in temperature (35 °C vs. ambient) makes the most sense in terms of signal/noise (Table 3.1).

Table 3.1. Signal and Noise vs. Temperature Setpoint on CC-5 Preheater Module.
Conditions: 2.2 mL/min, +750 mV/GC/RE-4 reference, reversed-phase ion-pair separation, norepinephrine is test solute.

Temperature	Peak Height	Noise	SNR
ambient	1.9	0.1	19
35	2.7	0.1	27
45	3.7	0.2	18
55	4.9	0.25	20

Section 4. Oxidative Mode LCEC

The oxidation of a compound involves the electron transfer from a molecule to the electrode surface. The two major advantages of most oxidative applications are (1) that oxygen is not electrochemically active, and (2) that solid electrodes can be used. Of course, both of these are definite advantages in the LCEC experiment as well. The importance in removing oxygen for reductive electrochemistry was discussed in Section 1, and the LC system modifications required for these determinations will be discussed in Section 5.

The working electrode materials most commonly used are based on a carbon matrix, typically an anisotropic solid such as glassy carbon. Obviously, other materials exist and can be used for the LCEC determination. For example, gold, nickel, silver, platinum, and a thin mercury amalgam can all be used for specific measurements.

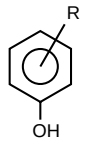
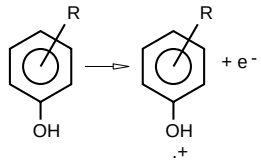
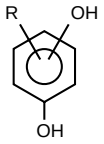
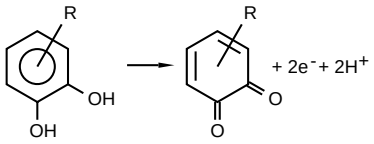
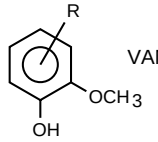
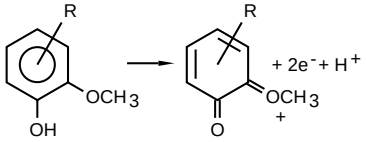
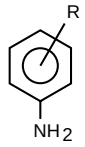
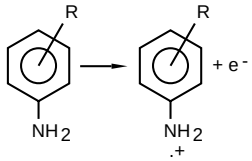
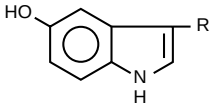
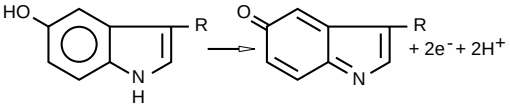
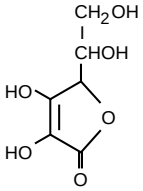
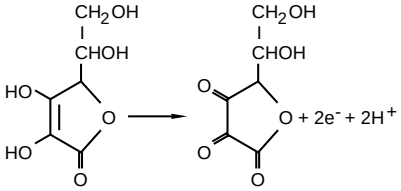
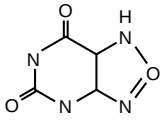
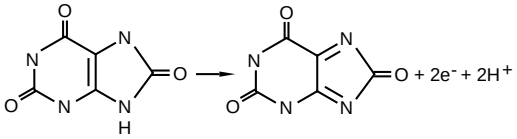
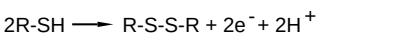
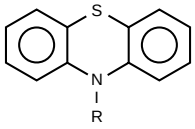
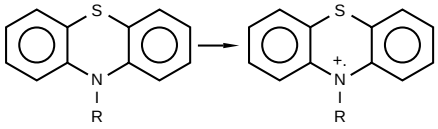
This section will primarily deal with typical functional groups that can be oxidized, but the list generated below is by no means exhaustive. The only conclusive way of knowing if a compound is electrochemically active is by a technique such as cyclic voltammetry. Even with our experience, we cannot always "guess" at how a specific compound may behave. A voltammetric assessment can provide information about the reversibility of the reaction as well, which can influence a decision on which mode of LCEC to use (e.g., Dual Series). In general, for electrochemical oxidation to take place, one can look for the same features and reactive centers as one would for a homogeneous oxidation reaction (e.g., delocalized electrons, stability of product, etc.). This does not mean that if a homogeneous chemical reaction does take place then this is directly applicable to the heterogeneous electrochemical reaction. The correlation is often thought to occur, and is many times an incorrect assumption.

4.1 Typical Functional Groups

The listing in Table 4.1 is not all-inclusive but only indicates some typical functional groups and compounds that are known to be electrochemically active. Later sections in this manual contain additional information based on cyclic voltammetry studies. If you have any questions regarding the electrochemical activity of your particular compound under a given set of conditions, it is best to do the voltammetry experiment.

Laboratories that are often investigating new compounds would find a BAS cyclic voltammetry instrument or the BAS 100B Electrochemical Analyzer a worthwhile investment.

Table 4.1. Functional Groups Suitable for Oxidative Electrochemical Detection.

ACTIVE FUNCTIONAL GROUP	CLASS	TYPICAL ELECTROCHEMICAL REACTION	EXAMPLES
	PHENOLS		PHENOL PENTACHLOROPHENOL PARABENS MORPHINE TYROSINE
	HYDROQUINONES		CATECHOLAMINES
	VANILLYL COMPOUNDS		HOMOVANILLIC ACID VANILMANDELIC ACID FERULIC ACID
	AROMATIC AMINES		ANILINE BENZIDINE
	INDOLES		TRYPTOPHAN SEROTONIN 5-HIAA
	ASCORBIC ACID		ASCORBIC ACID (VITAMIN C)
	XANTHINES		URIC ACID THEOPHYLLINE
R-SH	THIOLS		CYSTEINE GLUTATHIONE
	PHENOTHIAZINES		CHLORPROMAZINE

Section 5. Reductive Mode LCEC

This section details specific liquid chromatographic procedures for reductive mode LCEC. The section is divided into five parts:

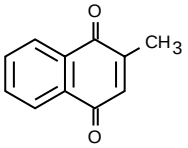
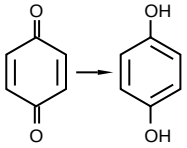
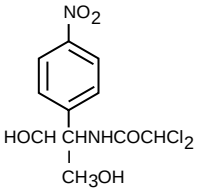
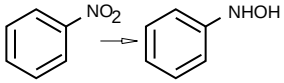
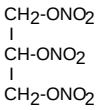
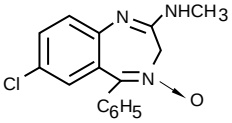
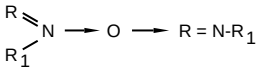
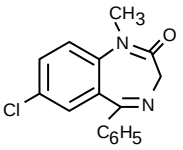
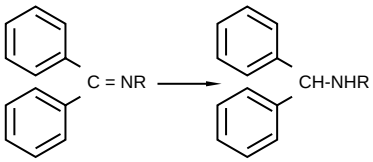
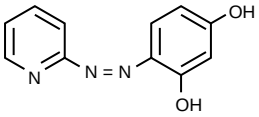
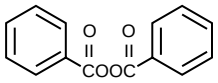
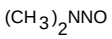
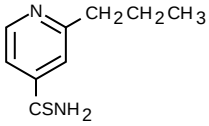
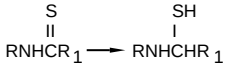
1. typical functional groups detectable by reductive LCEC,
2. system modifications required for reductive LCEC,
3. degassing procedures for mobile phase,
4. degassing procedures for samples, and
5. relative merits of glassy carbon and mercury/gold electrodes for reductive LCEC.

The preparation of both glassy carbon and mercury/gold electrodes is described in detail in the Electrode Polishing and Care Manual (A-1302) shipped with the detector accessories. Users with questions concerning the cell itself should read this material first.

5.1 Typical Functional Groups

By far the majority of the LCEC literature deals with oxidative mode electrochemical detection, but considering strictly the electrochemical literature of organic compounds, reduction processes have been examined in much greater detail. Table 5.1 describes some functional group candidates capable of being analyzed by reductive LCEC. Note that within a given functional group, a broad range of reduction potentials may exist due to the effects of substituent groups. Generally, the more delocalized the electrons become, the more easily reducible the substance. In addition, electron-withdrawing groups on an aromatic ring will enhance the reduction reaction.

Table 5.1. Functional Groups Suitable for Reductive Electrochemical Detection.

ACTIVE FUNCTIONAL GROUP	CLASS	TYPICAL ELECTROCHEMICAL REACTION	EXAMPLES
	QUINONES		VITAMIN K3
	AROMATIC NITRO		CHLORAMPHENICOL
	ALIPHATIC NITRO		NITROETHANE NITROGLYCERIN
	ORGANOMETALLICS		TRIPHENYLLEAD CIS-PLATINUM
	N-OXIDES		CHLORDIAZEPOXIDE
	AZOMETHINE		DIAZEPAM NITRAZEPAM
	AZO COMPOUNDS		(4-(2-PYRIDYLAZO)RESORCINOL)
	PEROXIDES		BENZOYL PEROXIDE
	NITROSAMINES		DIMETHYLNITROSAMINE
	THIOAMIDES		PROTHIONAMIDE

5.2 System Modifications for Reductive LCEC

Reductive mode LCEC requires some mechanical modifications to remove dissolved oxygen from both the mobile phase and sample; it is just as necessary to make sure oxygen does not reenter the system. Oxygen is removed from the mobile phase and sample by bubbling an inert gas (e.g., helium) through the solutions. Refluxing the mobile phase at the same time will thoroughly deoxygenate the system. A mechanical arrangement such as that shown in Figure 5.1 can be set up and dedicated to a specific LC system for this purpose. Table 5.2 details specific parts needed for this modification to modular BAS LCEC systems. All plastic tubing in the system must be replaced with stainless steel, because most plastic tubing is permeable to oxygen (PEEK tubing is less permeable than most, and may be an acceptable substitute for stainless steel). A one- or two-liter round-bottom flask with reflux condenser and heating mantle serves as the mobile phase reservoir. One neck is used for the condenser, and the other two necks for the helium inlet and pump outlet. Ordinary laboratory grade helium supplied at ca. 20 psi is tapped at two separate needle valves for independent control of both solvent and sample degassing. A 1/8"-o.d. stainless steel outlet line runs to the inlet check valve of the pump. Large rubber septa seal off the mobile phase from the outside environment. Temperature of the heating mantle can be controlled electronically.

Samples are degassed with the second needle valve. An optional helium saturation chamber filled with water wets the gas thoroughly before it enters the sample container.

The outlet tube from the column to the detector must be replaced with a special steel connector (P/N MF-1029). No plastic tubing may be used! This connector uses special plastic end fittings to electrically isolate the cell from the rest of the LC system (which is grounded). The steel tubing prevents gas intrusion. If you are using a preheater module, the steel tubing in this is appropriate.

Modifications are now completed for reductive LCEC.

If you think this seems like a lot of preparation, you are correct. After the initial setup, a system like this can be fairly reliable for reductive LCEC work, but it has limitations. For example, no gradient elutions can be run under these conditions. For customers requiring a full-featured liquid chromatograph with solvent deoxygenation capabilities, the BAS 200B Analyzer is the instrument of choice.

5.3 Procedure for Degassing the Mobile Phase

The following chromatographic startup procedure is recommended for reductive LCEC applications. Before initiating flow through the LC system, heat the mobile phase to 30–35 °C and bubble the inert gas rather vigorously through it for 30–60 minutes. Start pumping the mobile phase through the LCEC system during the last 15 minutes of vigorous bubbling. Even though the mobile phase is degassed, the entire system is not. Oxygen penetrates the stationary phase pores, and it must be flushed out of these pores by initiating the flow of degassed mobile phase through the LC system. This may take several hours.

Degassing of the system requires at least 100–200 mL of preheated mobile phase. Be liberal and flush thoroughly. Turn on the working electrode after flushing and keep the detector at the lowest gain (highest RANGE setting) until the background current has stabilized. Reduce the flow of helium through the mobile phase. Some flow must be maintained to keep the oxygen out, but this need not be as vigorous as during the initial deoxygenating. If the baseline response (background current) begins to gradually increase, the rate of bubbling inert gas through the mobile phase is insufficient to keep the oxygen out of the system and must be increased until the background stabilizes. NOTE: Valuable time can be saved if degassing of the system is performed overnight at a minimal flow rate (0.2–0.3 mL/min). You can turn on the working electrode before leaving the lab in the evening to provide you with a stable system in the morning.

5.4 Procedure for Degassing Samples

Sample degassing is necessary when working at potentials more negative than 0 to –0.1 V for mercury/gold and –0.3 to –0.4 V for glassy carbon electrodes, as illustrated by hydrodynamic voltammograms in Figure 2.2. The potentials at which the electrodes will be insensitive to dissolved oxygen may vary, depending on the pH, concentration and type of nonaqueous solvent, and the previous history of the electrode surface.

Care must be taken while degassing a sample in order to preserve its original composition. This is extremely important when handling volumes smaller than 500 µL. Presaturating helium gas with mobile phase and maintaining a gentle flow of helium gas through a sample will minimize evaporation of a sample. A presaturation device is pictured in Figure 5.1.

To degas, pass helium into the sample for about 3–5 minutes. This should be regulated at a flow rate as vigorous as allowed by the sample volume (smaller volumes will have to be degassed more gently than larger ones).

To inject, it is best to draw the sample slowly into the injection loop by gentle suction. Exposure to the atmosphere is avoided, and the integrity of the closed system, particularly at the needle seal, is preserved. On BAS chromatographs, you would immerse the waste port of the injection valve into the deoxygenated sample, and fill the loop by aspiration with a syringe mounted in the front ("sample injection") port.

Figure 5.1. Deoxygenation apparatus for LCEC; both mobile phase and sample are deoxygenated.

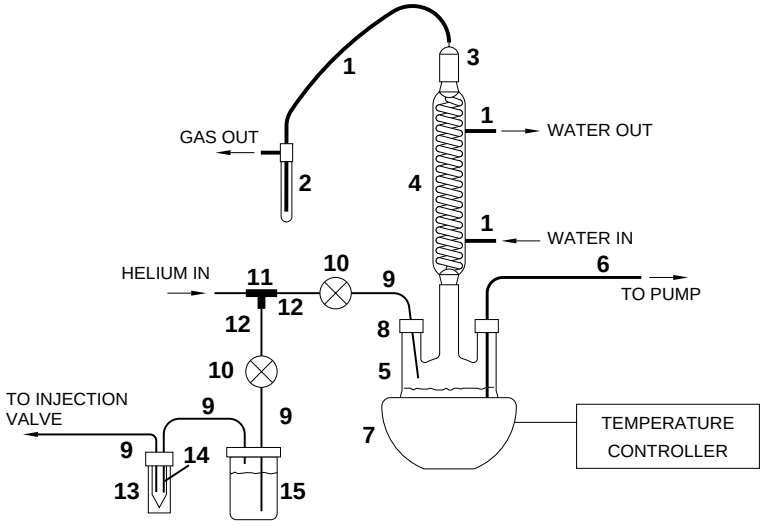


Table 5.2. Parts list for reductive LCEC.

Ref.	Description	Source
1	Connecting tygon tube	Common Lab Supplies
2	Gas-trap bubbler	Reliance Glass
3	Condenser adapter	Reliance Glass
4	Condenser	Reliance Glass
5	3-neck, 1 liter, round-bottom flask	Kimble
6	Stainless steel inlet tube for pump Model PM-80	BAS
7	Heating mantle	Scientific Products
8	Rubber septum	Common Lab Supplies
9	1/16" o.d. stainless steel tubing (when attaching to valve you may need extra fittings)	BAS
10	Needle valve	Swagelok
11	Tee to He line	Swagelok
12	1/8" o.d. copper tubing	Common Lab Supplies
13	Sample vial with rubber septum	BAS
14	Vent needle	Scientific Products
15	Gas humidifying chamber	Common Lab Supplies

5.5 Glassy Carbon Vs. Mercury/Gold Electrodes for Reductive LCEC

Both glassy carbon and mercury/gold electrode surfaces are useful in reductive LCEC. Although mercury is usually the surface of choice with electrochemists, there are fewer reasons for using it in LCEC. In polarography, mercury provides a repeatable, fresh electrode surface, a high hydrogen overvoltage (overpotential), and fast setting times. In liquid chromatography, however, dead volume must be minimized, thereby obviating the use of the usual dropping mercury electrode. For reductive LCEC, the optimal approach is to employ a thin-layer cell. The dropping mercury electrode is replaced by a glassy carbon surface or a mercury film.

To select the proper electrode, follow these guidelines:

1. For reductions requiring potentials between +1.00 and –0.90 V (vs. Ag/AgCl), try glassy carbon first. Glassy carbon offers better long-term stability than mercury/gold. Background currents should be less than 100 nA throughout this range. Usable performance may be obtained at more negative potentials, depending on the conditions.

2. For reductions at potentials between –0.90 and –1.1 V, a freshly prepared mercury/gold surface will probably be necessary for sufficient hydrogen overvoltage protection. Do not expect subpicomole detection limits, however! Remember, at these applied potentials, you are dealing with a high-energy situation (not unlike low-wavelength UV detection). Everything will be noisier and your detection limits will be hindered because of it.

3. Some applications will intimately involve the surface chemistry of one electrode material, thereby favoring that material. For example, although this is not an electrochemical reduction, the detection of sulfhydryls on mercury occurs at a potential about 600 mV less positive than on carbon due to the following mechanism:

   $$Hg + RSH \longrightarrow HgSR + e^- + H^+ \qquad E_p = +0.2 \text{ to } +0.4\ V$$

   $$2\ HgSR \longrightarrow Hg + Hg(SR)_2$$

   It is the complex of mercury and thiol that is actually undergoing the oxidation.

   On carbon, it has been shown that disulfides are the usual product, with the reaction taking place at a much higher applied potential:

   $$2\ HgSR \longrightarrow RSSR + 2H^+ + 2e^- \qquad E_p = +1.0\ V$$

4. If permitted under the constraints of guidelines 1–3 above, use glassy carbon for longer service lifetimes. A mercury/gold amalgam is a solid solution at the interface between the gold substrate and the thin mercury film; eventually the gold will diffuse through the film to the surface. The advantageous hydrogen overvoltage will eventually vanish.

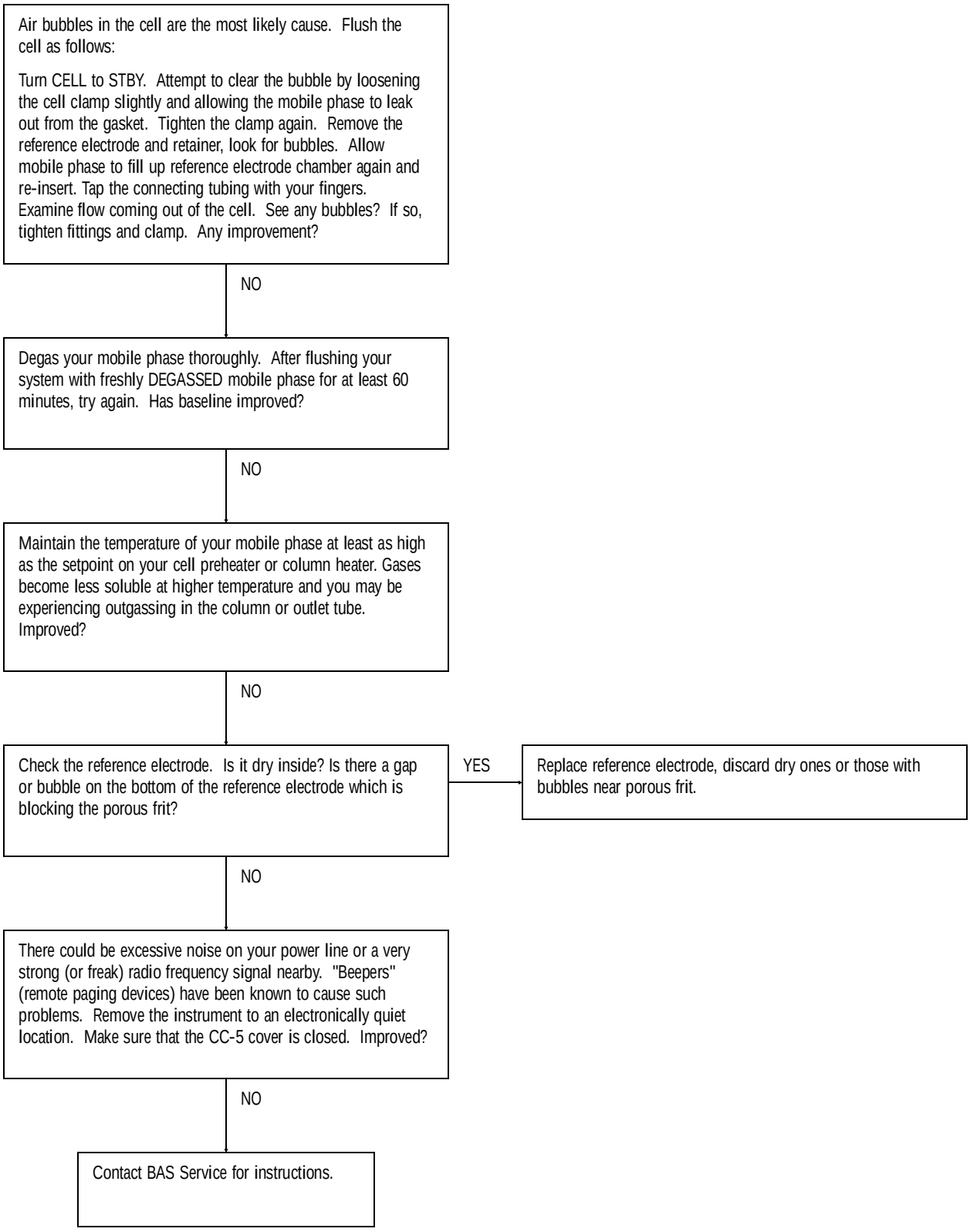
Section 6. Troubleshooting Flow Charts

The following series of charts will help you identify and correct the majority of problems you might encounter with your electrochemical detector.

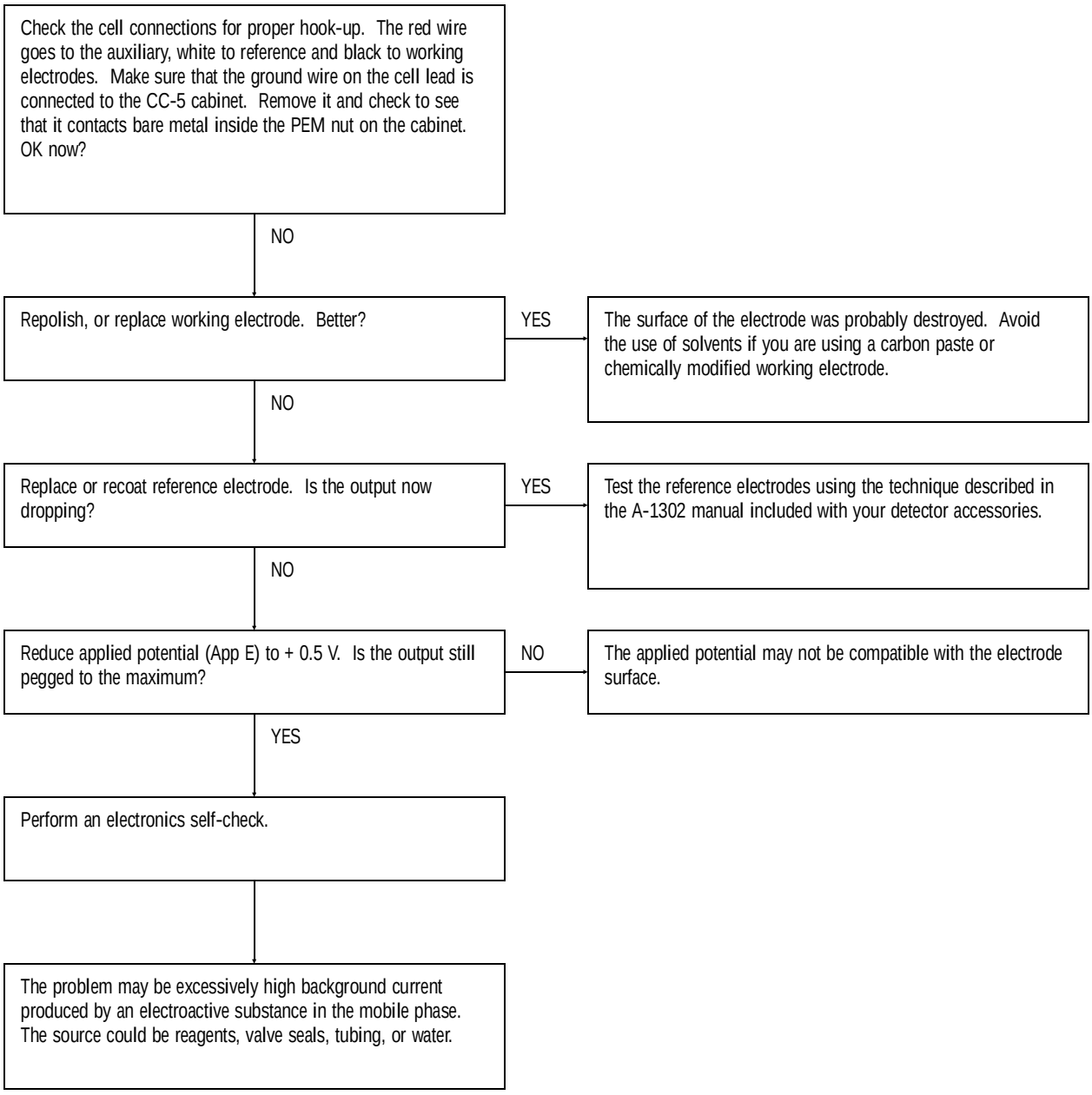
If these suggestions do not help you to solve the problem, please make note of the following information before you call for service and technical assistance. It will give us a more complete picture of what you are trying to accomplish.

Detector	Type of Detector?	Single Electrode, Dual Electrode
	Cell Preheater Installed?	Yes, No
	Working Electrode Type?	Glassy Carbon, Gold Amalgam, Paste, Silver, Platinum, Nickel
	Electrode Arrangement?	Dual-Series, Dual-Parallel, Single or Jumpered?
	Range?	_____ nA displayed on LC-4C front panel
	Offset?	_____ nA
	Output?	_____ nA full scale
	Applied Potential?	_____ Volts, + or –
	Oxidation or Reduction?	switch on back panel of LC-4C
Chromatography Conditions	Mobile Phase Composition?	_____
	Mobile Phase pH?	_____
	Column Type and Length?	_____
	BAS Liquid Chromatograph?	BAS 200, BAS 460, BAS 480, other _____
	Using with other detectors?	Yes, No
	Position of EC Detector?	upstream, downstream
Application	Sample Type?	biological fluid, tissue, perfusate, pharmaceutical, runoff water, cosmetic, extract, etc.
	Analyte of Interest?	_____
	Concentration in Sample?	_____
	Detection Limit Sought?	_____

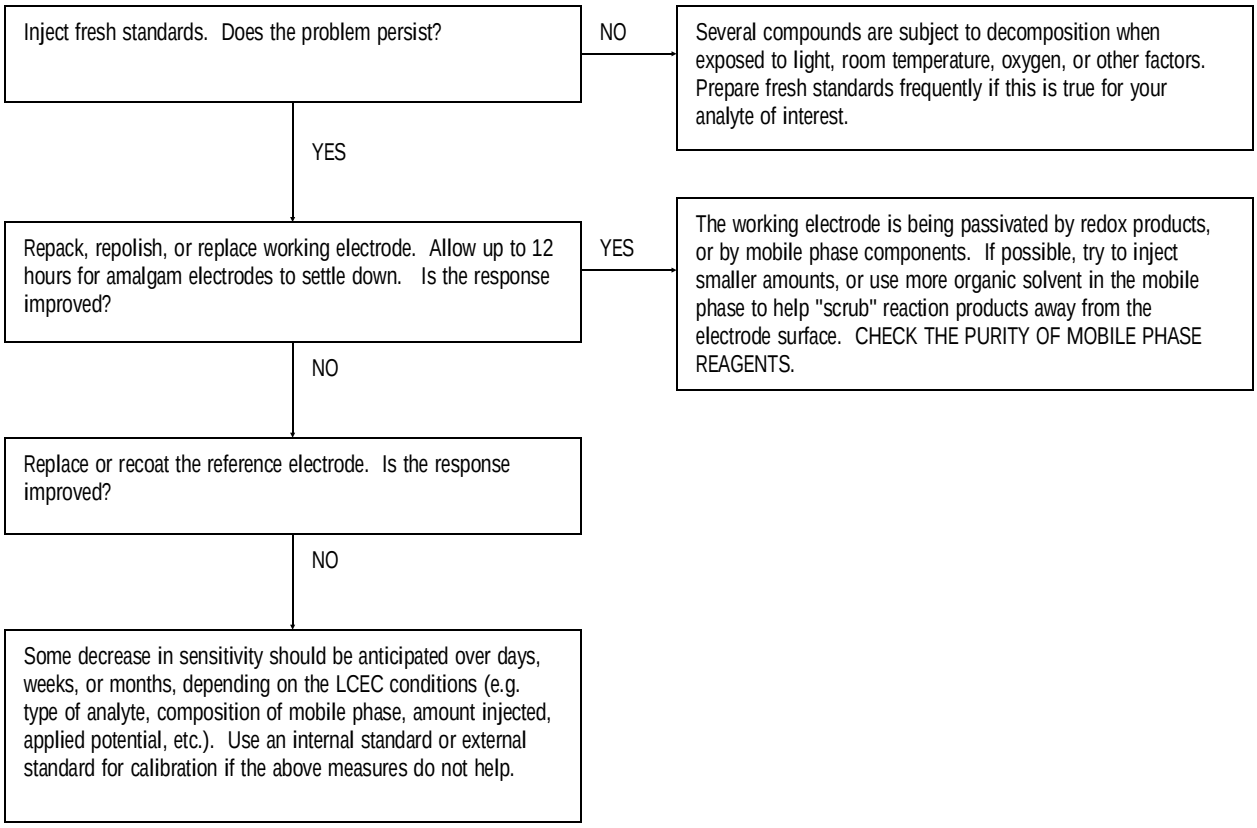
PROBLEM: VERY FAST NOISE SPIKES, PARTIAL OR FULL SCALE.



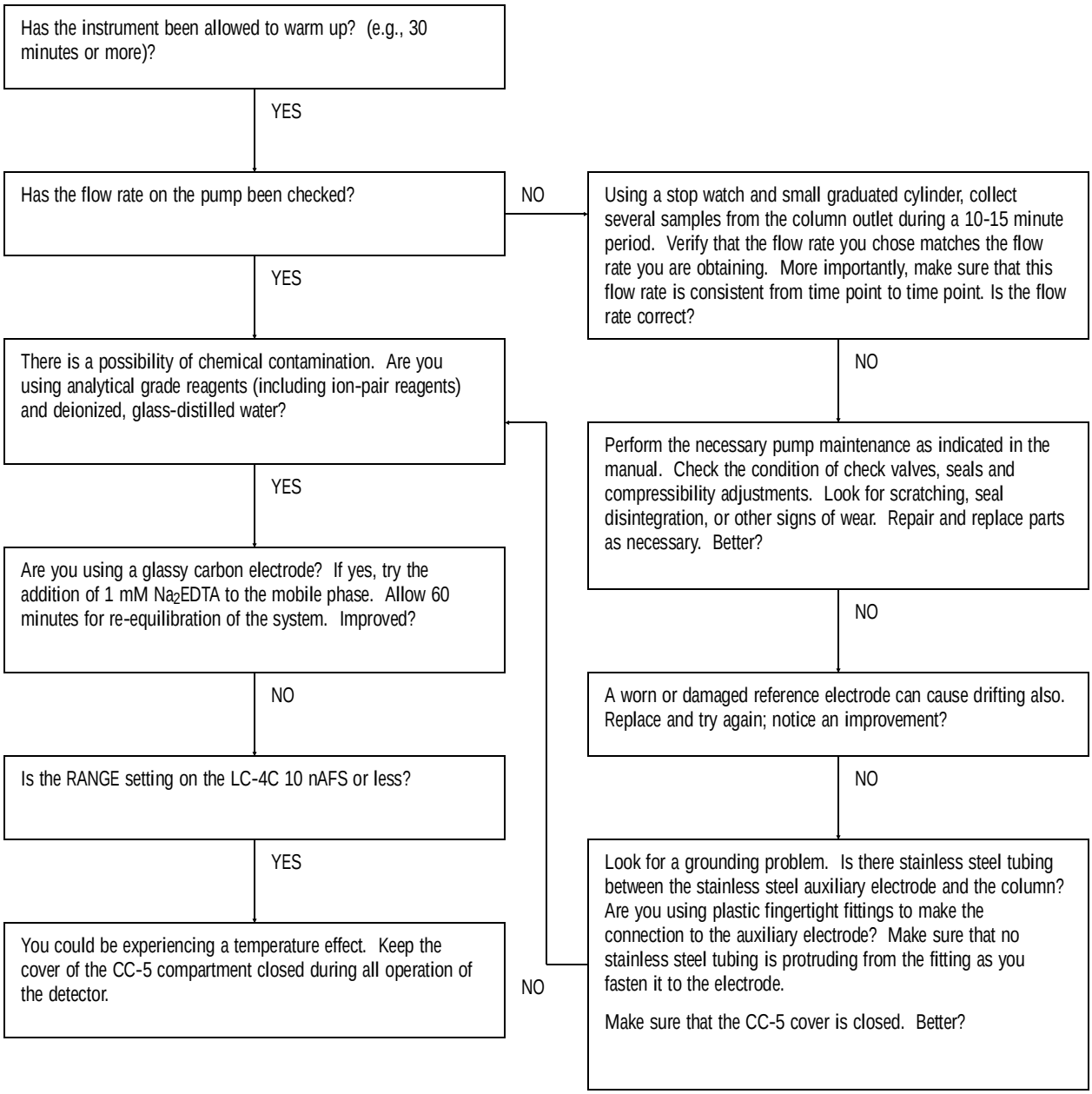
PROBLEM: DETECTOR OUTPUT IS CONSTANTLY PEGGED AT THE MAXIMUM LEVEL (120 mV or 12 V, DEPENDING ON THE OUTPUT JACKS SELECTED).



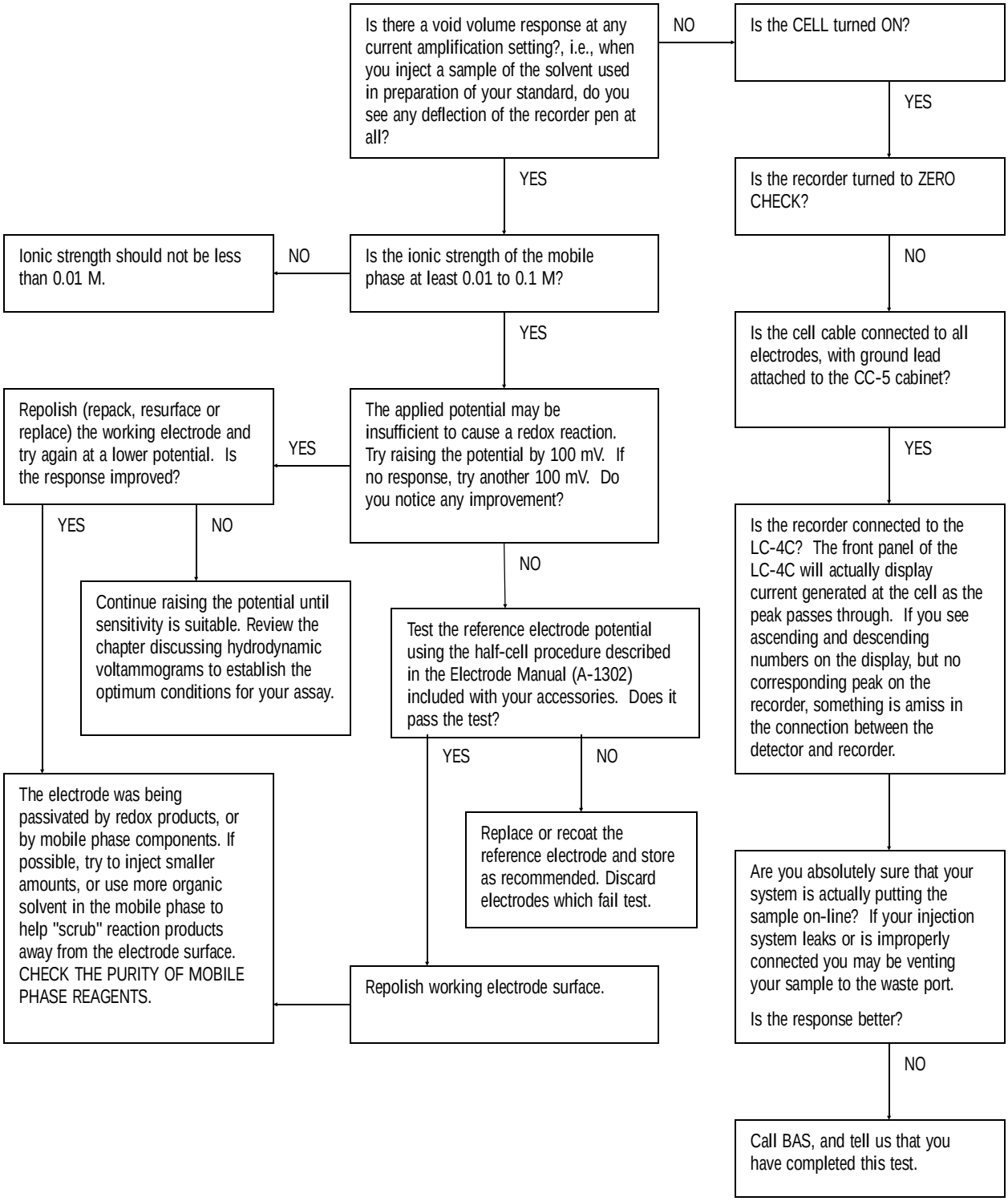
PROBLEM: ELECTRODE IS RESPONSIVE, BUT THE SENSITIVITY IS SIGNIFICANTLY LESS THAN IN PREVIOUS RUNS.



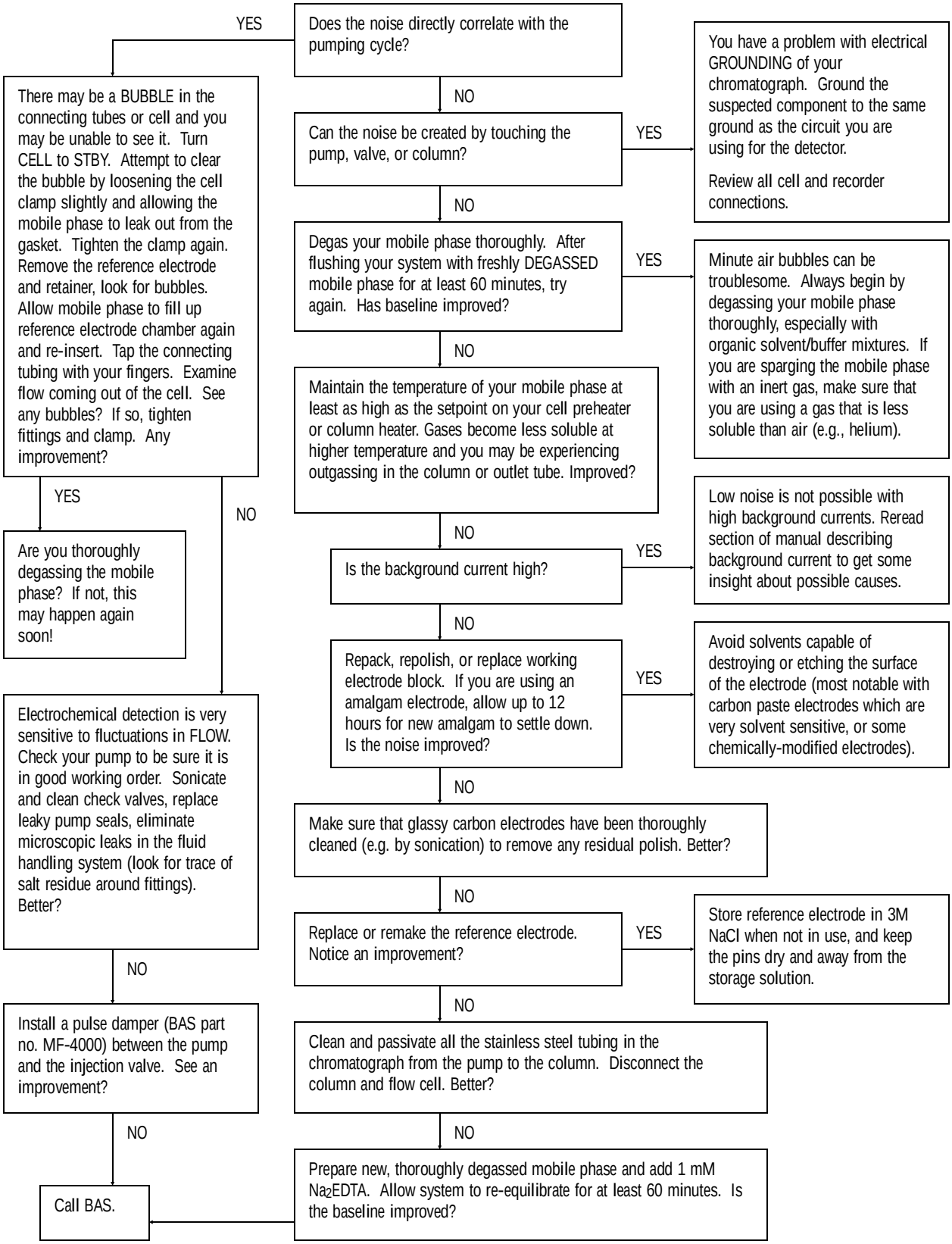
PROBLEM: IRREGULAR DRIFT IN BASELINE



PROBLEM: NO RESPONSE FROM DETECTOR



PROBLEM: BASELINE NOISE LIMITS SENSITIVE ANALYSIS



Section 7. Correcting High Background Problems

In cases in which high background current is limiting detector operation, make the following checks.

1. Select the highest quality buffer salts and solvents when making the mobile phase. Use distilled, deionized water.

2. Filter and degas the mobile phase.

3. Operate the detector at the minimum applied potential for your compound of interest. If you're not sure what this is, do an HDV or CV experiment (see Section 1).

4. Select an electrode that is appropriate for your mobile phase. Make sure it is clean and in good condition. Newly amalgamated mercury/gold electrodes may have to "equilibrate" overnight prior to being used.

5. Check that pump seals are in good working condition.

6. Check the electrical grounding of the system (especially if it exhibits a large response when a component is touched).

7. Eliminate the column from the flow stream and connect the system directly to the LCEC cell. If background drops (despite the high pulsations), clean or replace the column.

8. If all else fails, passivate the system following the procedure outlined below.

Passivation of the LC Components: Its Importance and Function

If you start pumping a freshly prepared mobile phase through your system and observe 50–75 nA of background, where normally you would expect 1–2 nA, you know you have a problem. It's probably caused by contaminant(s) in the newly prepared mobile phase. However, what if the background current only increased to 5–7 nA? Do you have a problem, or is this normal variation? If past experience suggests that there is a problem, then a low concentration of contaminant(s), column washout, corrosion, or bacteria may be the cause.

Nearly all liquid chromatography systems rely on stainless steel construction for the high-pressure connections and flowpaths. Of the "inert" materials available, stainless steel is one of the few that satisfies criteria relating to cost, machinability, strength, and availability. Plastics are now available for some high-pressure applications, but these are gas-permeable. This limitation represents a drawback in situations where oxygen must be avoided.

Passivation is a commonly recommended treatment for stainless LC systems. What is passivation, and why is it useful?

A metal may be designated passive if it corrodes at a very low rate during exposure to its chemical environment. Corrosion, the loss of metal to its environment by chemical attack, can be classified as either uniform or galvanic. The latter occurs when dissimilar materials in contact with one another create "batteries" of electromotive force capable of ruining their exposed surfaces. Even within an apparently homogeneous alloy of stainless steel, there exist many such "batteries," principally at microcrystalline grain boundaries not visible to the eye.

These boundaries are formed during creation of the bulk alloyed metal, and are usually accentuated by the machining processes used to create the finished part. Where the tool cuts the metal, localized "hot spots" occur. The alloy can thus become heterogeneous on an atomic scale. Surface crevices no longer resemble the parent alloy and can be attacked preferentially by corrosive chemicals. This intergranular corrosion can be attenuated by annealing the fabricated parts in a hot reducing atmosphere. The intent here is to redissolve these boundaries and to recreate a uniform alloy. Pitting, crevice, and erosion are other forms of corrosion that can diminish the integrity of an expensive LC system.

Passivation of stainless steel results in high corrosion resistance. Typically this involves immersion of the finished component in an oxidizing medium such as nitric or chromic acid. The corrosion rate may be greatly reduced—but not eliminated—by such treatment. Technically, the purpose of the acid is to clean the steel: remove iron atoms, inclusion impurities, and surface features that would serve as initiation sites for corrosion. Once clean, the active metal surface will spontaneously passivate in the air or in oxygen-containing solutions. All LC systems manufactured by BAS undergo this treatment prior to testing.

The elimination of unalloyed iron from these systems is critical for a successful LCEC determination. If iron is not masked by chelating agents such as EDTA, reactions such as

$$Fe^{2+}(aq) \longrightarrow Fe^{3+}(aq) + e^{-}$$

can occur at the anode and cause high detector background signals. Cleaning with oxidizing acids removes these pockets of unalloyed iron and simultaneously allows the new oxide layer to form.

The need to passivate a system may be ascertained by the following test. If a mobile phase normally contains 0.1–0.5 mM EDTA and corrosion is suspected because of increasing background and noise, then raise the EDTA concentration to 1–2 mM. An improved baseline will usually indicate that the system should be passivated for improved performance.

BAS has found that certain components are much more susceptible to corrosion than others. For example, we avoid tube fittings that have been "nitrided" for hardness. The same process occurs on the back ferrule of some fittings. The rust-prone area is easily visualized by soaking the ferrules in dilute HCl or HNO_3. In the BAS labs, a cause-and-effect relationship was hard to establish, since the ferrules were not directly in the flowpath. The symptom was slowly climbing background current, with an induction period of about 24 hours. The latter was apparently due to the time needed for the solvent to eddy into the ferrule zone and then eddy back out into the flowstream. Replacing the ferrules with non-nitrided parts eliminated the problem. Due to their extremely high surface areas, frits for columns and gas sparging also readily release trace amounts of iron. For this reason, the BAS 200B uses titanium frits for gas sparging.

A system that has been neglected or abandoned should be returned to service only after careful attention to passivation.

Passivation requires that 6 N nitric acid come in contact with all wettable surfaces of the LC system. Make sure these components are compatible with this process. Leaking pump seals and any rusted or leaking fittings should be replaced. Connect the waste line so that splashing does not occur (paint will be removed and metal surfaces corroded) and be sure to wear eye protection and protective outerwear.

1. Start by cleaning and degreasing the system. This is done to be certain that the nitric acid will wet the metal surfaces. The column and detector can be in-line for this step, but MUST BE REMOVED before passivation. Leave the injection valve in the inject position so that the stainless steel sample loop is passivated.

2. Clean the system by flushing with deionized water (> 30 mL), followed by 50% methanol (> 30 mL), and then 100% methanol (> 100 mL). A flow rate of 2 mL/min can be used for these and subsequent washings and passivation. Although methanol helps to clean and degrease the metal surfaces, flush the system with a nonpolar solvent such as methylene chloride or cyclohexane (> 100 mL).

3. Reverse the procedure (100% methanol to 50% methanol to water), ending with water. Don't forget to remove the column and detector cell before proceeding! Also, switch the waste container (or wash out all organic fluid; do not mix solvent with nitric acid).

4. Pump 6 N nitric acid through the system. BAS recommends using 300 mL, although some labs use as little as 30 mL. After the acid treatment, rinse all headspace that the nitric acid and its vapors could have contacted. A wash-bottle with a piece of pliable tubing attached to the spout works nicely. The 6 N nitric acid is a relatively dense solution, so use at least three separate changes of deionized water in the mobile phase reservoir (you will be able to see the nitric acid "falling out" of the tubing in the reservoir).

   You can confirm the washout of nitric acid by checking the pH of the effluent. When it is the same as the pH of the fresh rinse water, flushing has been accomplished.

5. The final step is to flush the system with 60 mL fresh mobile phase before attaching a washed column for equilibration.

If the high background and noise in the EC detector were caused by iron, a reduction in both will be observed. If they persist, then a problem remains with the mobile phase, the column, the detector itself, or the data recording device (or incorrect hookup of the latter two components).

Section 8. Electrochemical Characteristics of Selected Molecules

What Are Cyclic Voltammetry Data Charts?

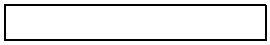
The following tables will help to summarize a great deal of information about the redox characteristics of a variety of molecules. This information can be used as a guideline when evaluating the feasibility of detecting these compounds using LCEC techniques. Information concerning the applied potential is not absolute; you will need to confirm the optimum using hydrodynamic voltammograms and the electrode and mobile phase necessary for your assay.

Format Of The Charts

The potential axis runs from left to right for reductions and from right to left for oxidations, per the traditional American convention.

Nomenclature

For REDUCTIONS: the left end of the rectangle represents the potential where the current is equal to 1/2 of the peak current. The right end of the rectangle represents the potential at the peak maximum. The opposite is true for OXIDATIONS.



An empty rectangle indicates that only one forward peak is observed and no other peak is obtained on the reverse scan between limiting potentials of the medium.

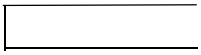
A shaded rectangle indicates that there is one reduction (or oxidation) peak and that an oxidation (or reduction) peak is observed when the scan is reversed. However, the electrochemical reaction is CHEMICALLY IRREVERSIBLE. For example,

$ArNO_2 + 4e^- + 4H^+ \longrightarrow ArNHOH + H_2O$
(Forward Scan = A Reduction)

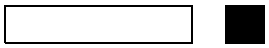
$ArNHOH \longrightarrow ArNO + 2e^- + 2H^+$
(Reverse Scan = An Oxidation, but not resulting in the original compound, and irreversible)

A solid rectangle means that the system is CHEMICALLY REVERSIBLE (and that the electron transfer system is sufficiently fast that both forward and reverse processes occur on the time scale of the CV experiment).

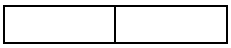
e.g., $MV^{+2} \underset{-e}{\overset{+e}{\longleftrightarrow}} MV^{+1}$



A rectangle without the right (or left for an oxidation) bar indicates that a poorly defined forward peak was obtained (a shoulder on the second wave) and that the $E_{1/2}$ value was estimated by setting $E_{1/2}$ at the potential equal to 1/2 of the current at the peak shoulder.



A solid bar following a rectangle indicates the peak potential of additional forward peaks that exist before the background limit. However, the $E_{1/2}$ value cannot be determined due to the poor resolution between adjacent peaks.



For differential pulse and other small-amplitude voltammetric techniques, this symbol is used. The central vertical bar represents the peak potential and the box indicates the width of the curve at half the peak current. The same symbol is used for polarography and hydrodynamic voltammetry where the three vertical bars indicate the $E_{1/4}$, $E_{1/2}$ and $E_{3/4}$ potentials. Again, a narrow box suggests a fast heterogeneous electron-transfer rate. An asymmetric box may indicate complications in the mechanism.

Electrochemical Characteristics of Selected Molecules of Biomedical and/or Environmental Interest

All cyclic voltammograms used to obtain data on the following 9 figures used identical conditions:

Electrolyte:	0.1 M Citrate, 10% Ethanol (V/V)
Scan:	200 mV/sec
Working Electrode:	Carbon Paste type CPO (for oxidative CV)
	Mercury/Gold Amalgam (for reductive CV)
Conditions:	Deoxygenation of solution was required for Reductive CV

Figure 8.1. Oxidative Cyclic Voltammetric Data for Selected Aminophenols.

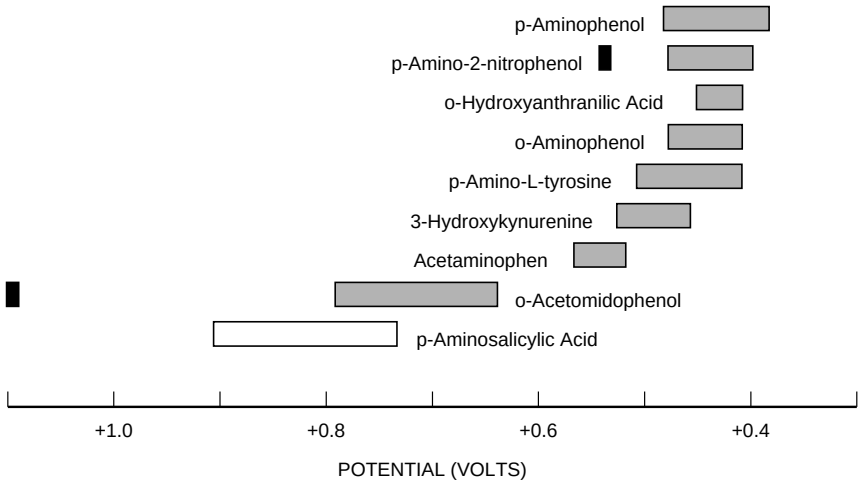


Figure 8.2. Oxidative Cyclic Voltammetric Data for Selected Aromatic Amines.

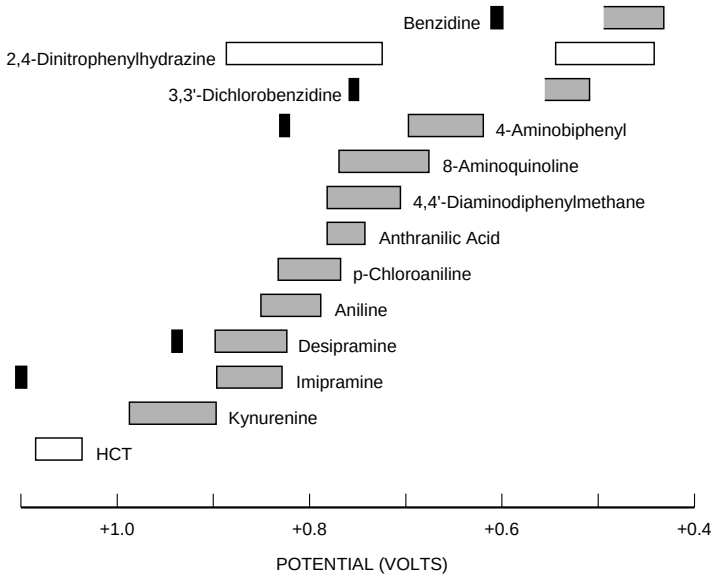


Figure 8.3. Oxidative Cyclic Voltammetric Data for Selected Alkylphenols of Environmental Interest.

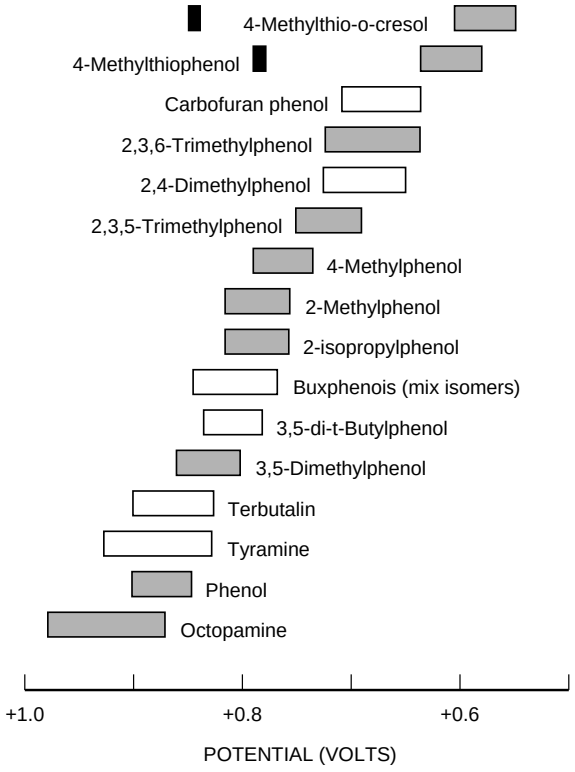


Figure 8.4. Oxidative Cyclic Voltammetric Data for Selected Chlorophenols of Environmental Interest.

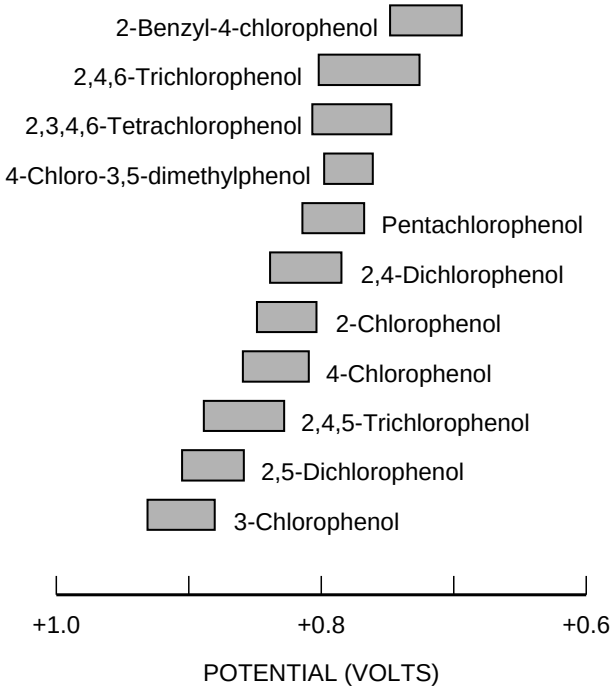


Figure 8.5. Oxidative Cyclic Voltammetric Data for Some Vanillyl Metabolites of Tyrosine and Related Compounds.

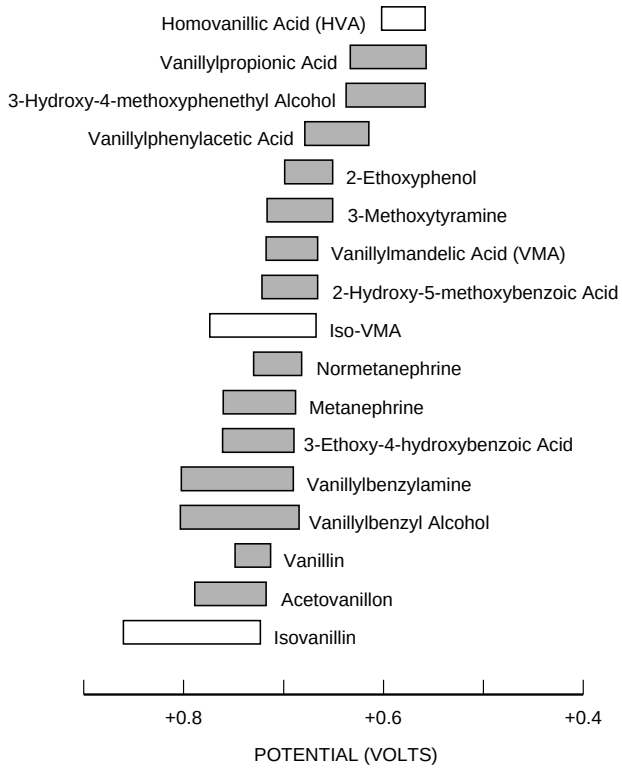


Figure 8.6. Oxidative Cyclic Voltammetric Data for Some Indole Metabolites of Tryptophan and Related Compounds.

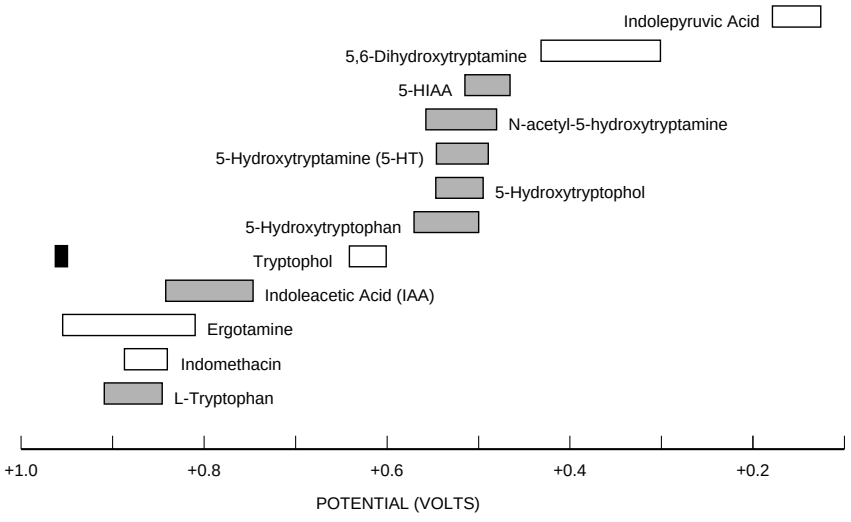


Figure 8.7. Oxidative Cyclic Voltammetric Data for Biologically Important Catecholamines and Other Easily Oxidized Diphenols.

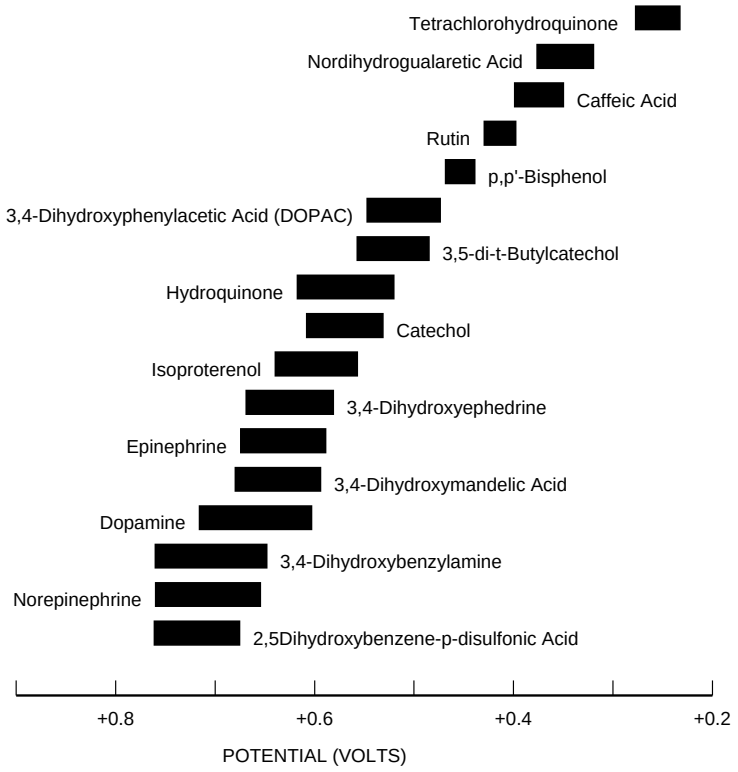


Figure 8.8. Oxidative Cyclic Voltammetric Data for Some Natural Phenolic Acids.

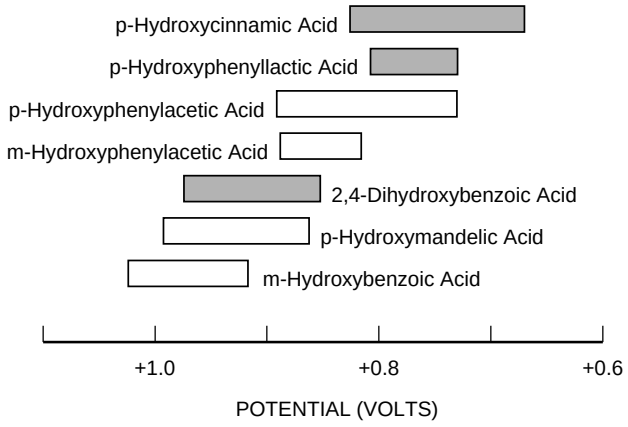


Figure 8.9. Reductive Cyclic Voltammetric Data for Selected Compounds of Environmental Interest.