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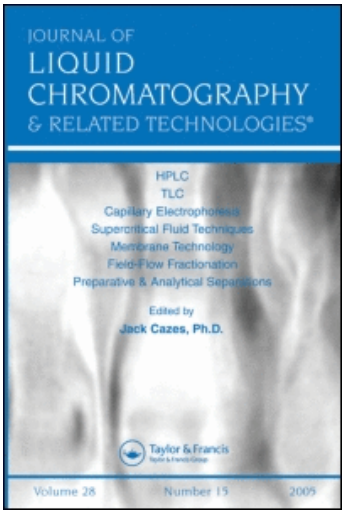

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Reductive Lcec of Organic Compounds

Karl Bratin[a]; P. T. Kissinger[a]
[a] Department of Chemistry, Purdue University, West Lafayette, Indiana

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REDUCTIVE LCEC OF ORGANIC COMPOUNDS

Karl Bratin and P. T. Kissinger
Department of Chemistry
Purdue University
West Lafayette, Indiana 47907

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ABSTRACT

Liquid chromatography and electrochemistry have been combined (LCEC) to form a new analytical approach with unique capabilities. While LCEC is now well established for oxidizable substances, its use for determination of reducible compounds is just beginning to develop. The electrochemistry of easily reducible functional groups (electrophores) is briefly discussed. Factors which influence the performance of reductive mode detection are reviewed, including mobile phase composition, electrode material, voltage waveform, and dissolved oxygen. Recent applications of reductive mode LCEC are presented.

I. INTRODUCTION

Progress in theoretical electrochemistry has been rapid over the past thirty years; however, a completely satisfactory physical and chemical interpretation which accounts for differences in electrode reactivities (overpotentials) is still not well understood. DC polarography and related voltammetric techniques have been used for the determination of literally thousands of environmentally and biologically important compounds. These techniques are very sensitive and reasonably selective, provided that the sample is not very complicated.

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Unfortunately, samples of biological, environmental, and clinical interest are usually complex and often require isolation steps prior to electrochemical determinations. As a result, classical voltammetric techniques have for the most part remained an academic curiosity.

The combination of an isolation (separation) technique with electrochemical detection in an "on line" arrangement has given electrochemistry a new following as a practical analytical tool. The benchmark paper which gave this field momentum demonstrated the virtue of combining high efficiency liquid chromatography with thin-layer hydrodynamic amperometry (LCEC) to detect easily oxidized compounds at solid electrodes (1). Recent technological advances and applications in this area have been thoroughly reviewed (2-6).

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Progress in reductive mode LCEC has been slow because, until recently, it has been difficult to prepare a reliable transducer which could be used with high efficiency liquid chromatographic separation techniques. The necessary requirements for such a transducer include low dead volume (20 μL), good tolerance to high volume flow rates (2-3 mL/min), chemical inertness to common solvents, easily renewable surface, and mechanical stability. The first section of this review considers some common electrochemical reactions which have been studied at mercury electrodes. A reasonable understanding of these reactions will be helpful for optimization of LC with reductive or dual-electrode transducers. Subsequent sections of the review consider the importance of the mobile phase, the electrode material, and the way in which the detector potential is tuned. Techniques for removal of dissolved oxygen and an overview of recent applications concludes this report. Reductive mode LCEC is developing very rapidly and the references in this review will soon be dated. Nevertheless, much of the material presented is of an introductory nature and should be of more permanent value.

II. ELECTROCHEMISTRY OF CANDIDATE ANALYTES

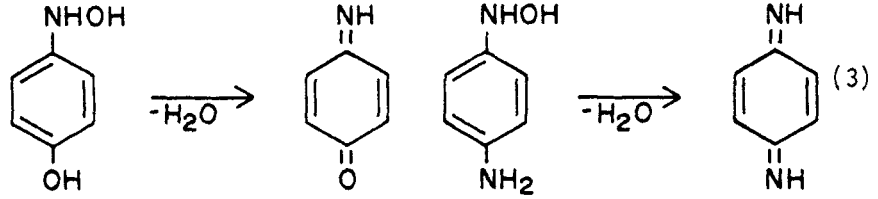
Electrochemical techniques have been useful in solving numerous problems in organic and inorganic redox chemistry. The following section briefly describes the electrochemistry of some of the key organic functional groups most often encountered in biologically and environmentally important compounds. Many other functional groups are electrochemically active at very negative potentials and thus are not amenable to direct LCEC determinations at favorable detection limits.

Nitro Compounds. The redox chemistry of nitro aromatic compounds has attracted considerable attention. The nitro group is an excellent electron acceptor. Its redox properties (e.g. reduction potential, number of electrons transferred) are strongly influenced by the nature and the position of other functional groups. Electrochemical studies on a variety of mono and polynitro aromatic compounds reveal that the reduction of the nitro aromatic group is irreversible and usually occurs in two steps to form an amine.

$$R\text{-}NO_2 + 4e^- + 4H^+ \rightarrow R\text{-}NHOH + H_2O \quad (1)$$

$$R\text{-}NHOH + 2e^- + 2H^+ \rightarrow R\text{-}NH_2 + H_2O \quad (2)$$

Compounds such as p- and o- nitroanilinesand p- and o-nitrophenols, which are capable of rapid dehydration of the hydroxylamines to form quinoid forms, (II and IV) undergo a single $6e^-$ reduction process, since the quinoid forms are easier to reduce than the parent nitro compounds.



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The reduction processes of polyaromatic nitro aromatic compounds are much more complex and several reviews have been published (7-9). The mechanism depends on the number of nitro groups and their relative position on the ring, the nature of other substituents on the aromatic system, and the pH. Typically, meta polynitro isomers with or without any alkyl substituents undergo sequential four electron reduction of each nitro group followed by a partial or complete reduction of the resulting hydroxylamine groups depending on the pH. Trinitrotoluene (TNT) is reduced in this fashion in both static solution and flowing streams, while picric acid is reduced by an initial six electron reduction of one nitro group followed by a partial reduction of the other two nitro groups ($10e^-$) (10).

Nitrate esters such as pentaerythritotetranitrate (PETN), isosorbidedinitrate (ISDN), nitroglycerin (NG), erythritoltetranitrate (ENT), and mannitolhexanitrate (MHN) are potent vassodilators. They are widely used to decrease the frequency and severity of angina attacks, decrease heart workload and reduce myocardial consumption of oxygen in patients suffering from various cardiovascular ailments. The nitrate ester functionality is reduced via a two electron process which releases nitrite ion and alcohol (11) as follows:

$$RO\text{-}NO_2 + 2e^- + H_2O \rightarrow ROH + OH^- + NO_2^- \quad (4)$$

Polynitrate esters undergo a stepwise reduction of each ester group according to the process given in equation 4.

Nitrosamines. In the past several years over 100 N-nitroso compounds have been found to be either carcinogenic or mutagenic to laboratory animals and humans. They have been found in various products, including cutting fluids, cosmetics, drugs, fish, and cooked cured meats.

The electrochemistry of the N-nitroso group has been studied in some detail by several investigators (12-14). At low pH, the

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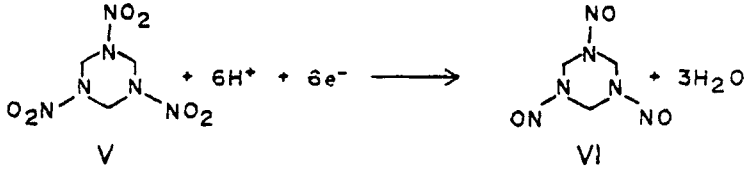
protonated N-nitroso group is reduced in a four electron process to form a hydrazine.

$$RR'NN^{+}OH + 4e^{-} + 4H^{+} \rightarrow RR'NN^{+}H_3 + H_2O \quad (5)$$

At neutral and alkaline pH, the N-nitroso group is cleaved to form dinitrogen oxide and an amine.

$$RR'N\text{-}NO + 2e^{-} + 2H^{+} \quad RR'NH + 1/2\ N_2O + 1/2\ H_2O \quad (6)$$

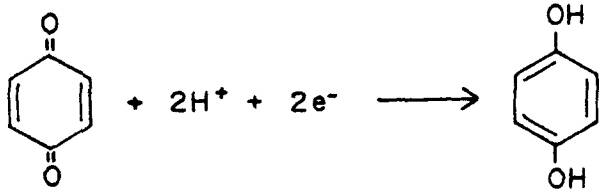
Nitramines. While there is a great wealth of information on the redox properties of nitro aromatic and nitrate esters, the opposite is true for nitramines. A single six electron reduction wave for RDX (V) forms the N-nitroso derivative (VI) which can undergo further reduction according to the process given by equation 5 (10).



V VI (7)

Quinones. A number of biologically important compounds contain the quinone structure including a group of naturally occurring substances referred to as the vitamin K's, which take an active part in the electron transport system and are involved in the formation of blood clotting factors.

The redox properties of quinones is well established. They are easily reduced to hydroquinones by a familiar two electron chemically reversible process:



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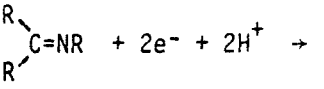
Imine, Azo, and N-oxide Functional Groups. The imine bond can be found in numerous industrial and pharmaceutical products and it is a characteristic functionality of 1,4-benzodiazepines, one of the most widely prescribed classes of drugs in the U.S. An "activated" (by aromatic system) imine bond undergoes a two electron reduction to form a secondary amine (15).



(9)

Compounds containing an azo group are often used as food additives and in commercial dyes and thus have a high probability of being found in biological fluids or the environment. An azo group is easily reduced in a two electron process to form a hydrazine (eq. 10), or in certain cases the reduction results

(10)

in the cleavage of the nitrogen double bond ($4e^-$) to form the corresponding amines (16).

N-oxides are typically encountered as metabolites of nitrogen-containing substances (e.g. imine, azo, and nitrogen heterocycles). N-oxides are reduced at relatively high negative potentials unless they are "activated" with an electron rich group such as a phenyl ring. At low pH, the protonated N-oxide bond is cleaved via a two electron process (17) as follows:

(11)

III. MOBILE PHASE CONSIDERATIONS

The usefulness of reductive mode electrochemical detection to a given problem ultimately depends on the voltammetric characteristics of the analyte in a suitable mobile phase and at a specific electrode surface. All detectors limit mobile phase

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composition to some degree; however, in electrochemical detection one must be aware of the fact that electrochemical reactions are strongly influenced by the physical and chemical nature of the mobile phase. The column and detector must be considered as a unit in selecting the mobile phase for optimization of LCEC determinations. While optimization of the mobile phase for reductive LCEC may not always be straightforward, useful generalizations can be made.

The majority of LC separations performed today employ reverse phase methodology. The mobile phases of choice are typically aqueous solutions with and without buffers and polar non-aqueous solvents (e.g. methanol, tetrahydrofuran, and acetonitrile). Depending on the physical properties of the analyte of interest, retention may be varied by adjusting the concentration of organic modifier(s), pH, ionic strength, or by adding ion-pairing reagents. At the present time, reverse-phase chromatography offers the best compatibility with electrochemical detection due to the polar nature of the mobile phase. Electrochemistry demands that the mobile phase conduct current (charge), be chemically and electrochemically inert at the selected detector potential and dissolve the analyte of interest. Direct electrochemical detection is not likely to be useful in normal phase chromatographic separations since nonpolar solvents are not very well suited to conduct electric current (have low dielectric constants) resulting in poor potential control. Totally nonaqueous mobile phases can be used with the electrochemical detector but require expensive salts (e.g. tetraalkylammonium hexafluorophosphates). These mobile phasesare especially useful when attempting to detect an analyte difficult to reduce, because they offer wider negative potential limits which vary depending on the electrode material (as discussed in section IV). This advantage is only achieved after tedious and time consuming removal of water from the nonaqueous solvent

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because the reduction of hydrogen ions limits the useful potential range of the electrode. The need for removal of water also complicates processing of samples and thus reductive LCEC in aprotic media has not achieved much attention.

Care is needed in selection of the mobile phase pH because it may strongly influence the electrochemical reaction of interest, the magnitude of the background current, and the LC separation process. This is particularly true in organic electrochemistry, where a generalized electrochemical reduction of an oxidized species (O) can be written as follows:

$$O + ne^- + nH^+ \rightarrow OH_n \quad (12)$$

In unbuffered mobile phases, pH changes near the electrode surface may have an adverse effect on the electrochemical processes. In acidic solutions the negative background limit is caused by evolution of hydrogen gas.

$$2H^+ + 2e^- \rightarrow H_2 \quad (13)$$

This shifts by approximately 59 mV/pH unit to more positive potentials with decreasing pH. At a given detector potential the background current (due to H^+ reduction) will increase with decreasing pH, thus decreasing the "operating potential window" of the detector and resulting in less satisfactory detection limits. The opposite effect is observed with ease of reduction. Since the majority of electrochemical reductions of organic compounds involve a transfer of a proton(s), it is easier to reduce molecules at lower pH (18). This means that less negative potentials (lower background currents) can be used for detection of the analytes of interest. Usually a compromise must be made in selection of the pH of the mobile phase in order to achieve the necessary separation and detector performance.

The choice and the concentration of buffer components may also have a significant influence on the performance of the

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electrochemical detector. The redox chemistry of analytes can be altered with different buffers due to specific analyte-buffer interactions (often observed with borate ions which are good complexing agents) or buffer-electrode interactions. Buffer solutions of moderate concentrations (e.g. 0.01 to 0.1 M) are generally used in LCEC. This is adequte to satisfy both the buffering and ionic strength requirements, especially under low current conditions. In reductive mode LCEC it is often desired to use mobile phases with lower buffer concentrations (on the order of 0.001 to 0.01 M) in order to minimize the concentration of transition metal ions (e.g. Pb^{+2}, Zn^{+2}, Cu^{+2}) in the mobile phase. These metal ions are easily reduced and deposited on the electrode surface. The resulting effect of metal deposition is a decrease in the negative background limit due to a lower hydrogen overvoltage. Traces of metal ions can be removed by preelectrolysis of the mobile phase (a procedure which can be cumbersome and time consuming for large quantities of mobile phase). Even though lower background currents are obtained with preelectrolyzed mobile phases, the real improvement in S/N and long term electrode stability was inconclusive for one series of experiments with a mercury film electrode (19), while Hanekamp and coworkers obtained a tenfold decrease in baseline noise with an on-line electrochemical scrubber (removed oxygen and metal ions) (20). The significant reduction in the baseline noise was probably achieved due to removal of dissolved oxygen.

The relatively large cathodic background currents and high solution resistance of low ionic strength nonaqueous mobile phases can limit the linear range (on the high end) of thin layer amperometric detectors (Figure 1A) due to uncompensated potential losses (a product of current and solution resistance, referred to as "IR drop") along the thin-layer channel (21).
Positioning the auxiliary electrode in the thin-layer channel directly opposite the working electrode (Figure 1B) reduces the

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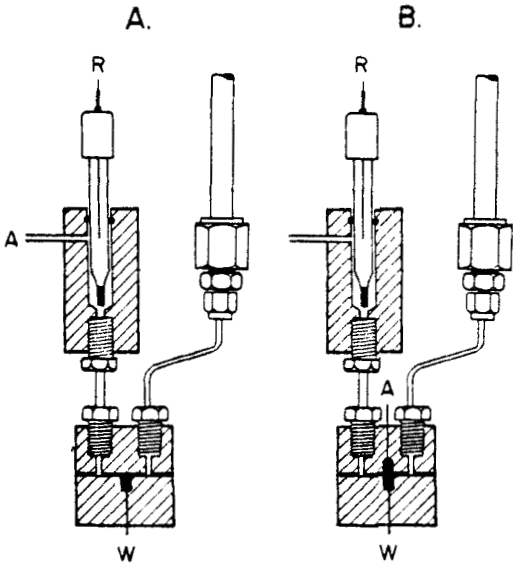

FIGURE 1. Common designs for the thin-layer amperometric transducer.

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IR drop to a negligible value resulting in extension of the linear dynamic range by at least one and a half orders of magnitude (to 5 1/2 orders of magnitude). This cell allows the use of nonaqueous and low ionic strength aqueous mobile phases because solution resistance across the thin-layer channel is negligible.

IV. CHOICES OF ELECTRODE MATERIALS

Several factors must be considered when selecting the electrode material of choice for reductive mode LCEC. The operating potential range or "window" of an electrode material is usually limited by the solvent breakdown. The limiting reaction in acidic solutions is hydrogen evolution, and in alkaline media the reduction of alkali metal ions is the limiting factor. The operating range of an electrode material

is often further limited by reduction of easily reducible impurities present in LC mobile phases (e.g. transition metal ions, organic compounds, dissolved oxygen) or by adsorption of compounds on the electrode surface. For an electrode material to be useful for reductive mode detection, it should have a low exchange current for hydrogen reduction (high hydrogen overpotential), good mechanical stability, and an easily renewable surface.

The most widely used electrode materials in electrochemistry are mercury drops (dropping and hanging), mercury films, amalgams, various forms of carbon, platinum, gold, silver, carbides, lead, nickel, borides, and semiconductor electrodes (e.g. SnO_2, TiO_2).

Mercury is the material of choice for most electrochemical reductions because it has a very large hydrogen overpotential and a smooth and easily renewable surface (22,23). Mercury pool electrodes are generally not suitable as an electrode material in a thin-layer transducer for LC because they suffer from vibrations and edge effects due to creeping of solution between the mercury and its container. While these problems are serious at low current densities (high sensitivity), mercury pool electrodes have been successfully used for the determination of sulfhydryl compounds (24,25).

The dropping mercury electrode (DME) LC detector was introduced in 1952 by Kemula (26), but its use was limited to a few academic laboratories due to the time dependent surface area, the expense and toxicity of mercury, the poor tolerance to a rapidly moving effluent, irregular drop size and drop time, the considerable charging current for a DME in a flowing stream, and awkward cell construction. Recent improvements in the design of the DME detector has stimulated renewed interest. Improvements in tolerance to a moving solution and reduction of the detector volume was achieved by means of a rapidly dropping mercury electrode (RDME) with a conically ground capillary (20-50 ms

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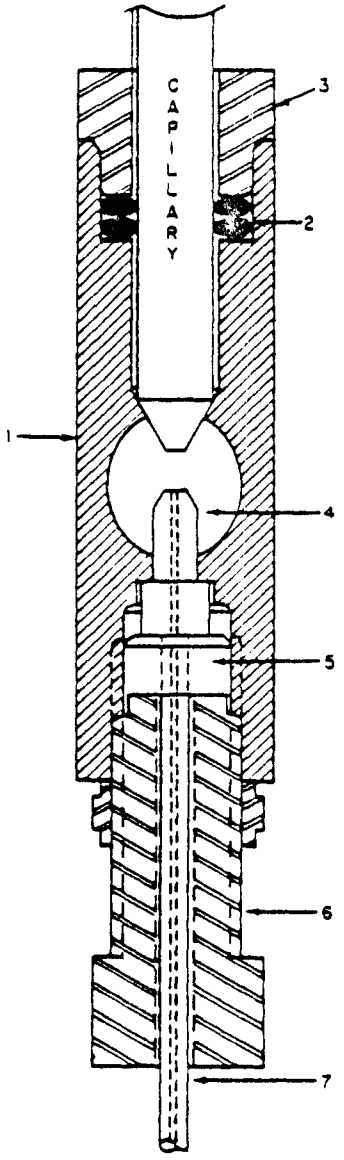

FIGURE 2. A schematic diagram of SMDE detector. (1) detector body, (2) o-ring, (3) plug, (4) flow adapter, (5) gripperfitting, (6) tubing endfitting, and (7) tubing. (Reproduced by permission of EG & G Princeton Applied Research Company.)

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drop times) (27-29) and a static drop mercury electrode (SMDE) (30). A schematic diagram of the SMDE is illustrated in Figure 2. Problems with "tear-off effects" on a RDME were eliminated with a straight flow channel (28). Nevertheless, even though several problems are serious (e.g. irreproducible drop sizes with small drops, loss of electrical contact due to early fall of mercury drops, collection of used mercury), several promising applications of the DME-based detectors have been reported and are summarized in the applications section of this review. Interest in DME-based LCEC transducers will continue because mercury has remained unchallenged in its negative "potential window" and constantly renewed surface which eliminates many problems arising from adsorption of organic compounds. Furthermore, mercury transducers (DME and SMDE) with differential and square wave detection modes will gain in popularity because the mercury surface is not susceptible to slow faradaic changes at the electrode surface (caused by reorganization of surface functional groups) which are very common for carbon electrodes.

Because problems with DME-SMDE LC transducers persist, several groups have concentrated on the development of suitable solid electrode transducers. The most popular designs have been the thin-layer transducers with flow parallel to an electrode(s) surface which is embedded in a cell block (Figure 1). Other designs include tubular electrodes (31-33), wall jet (34), carbon cloth (35,36), and a rotating disk electrode (37). While most of these have been developed for oxidative LCEC, some are also suitable for reductive LCEC.

The most commonly used electrode materials in LCEC are carbon paste (mineral oil base) and glassy carbon. Carbon paste is made by mixing an ultrapure graphite powder with mineral oil (or another dielectric material such as wax or silicone grease) to obtain a paste. The major advantage of paste electrodes is the relative freedom from surface filming. Usually electrodes

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will last for several days or weeks without resurfacing, depending on the electrode potential and type of samples injected. A serious disadvantage of most paste electrodes is their limited solvent compatibility. Mobile phases with more than 20-30% methanol (or acetonitrile) will dissolve the paste over a short period of time. Carbon paste exhibits a small negative limit due to persistent residual (background) currents. This background current has been attributed to the presence of oxygen dissolved in the paste or adsorbed on the electrode surface.

Glassy (or "vitreous") carbon electrodes (GCs), are prepared at elevated temperatures and pressures and are highly impervious to liquids and gases. They are inert to common organic solvents and are relatively free of entrapped gases and metallic contaminants. GC has the widest useful potential range in aqueous solutions (+ 1.2 to -0.8V) (38,39) among the common solid-electrode materials, so that both easily oxidizable and reducible organic species can be detected with these electrodes. A wider negative range has been reported by Lund and coworkers (-1.3V) (40); however, other workers have demonstrated that at potentials greater than -0.8 to -0.9V GCs become unstable resulting in poorer detection limits (39,40). The GCs in current use are subject to adsorption and may have to be polished frequently. The material has a reputation for a poor lot to lot reproducibility and long equilibration times at negative potentials, which is probably due to slow reduction of surface functional groups. Furthermore, faradaic reorganization of functional groups at the electrode surface, especially on GC, is often a slow process which wastes charge and leads to very large background and noise currents (poorer S/N ratios) when trace analysis is attempted.

One of the most important advantages of GC relative to mercury is a much larger oxygen overpotential (39) as illustrated in Figure 3. The large overpotential of oxygen on a GC allows

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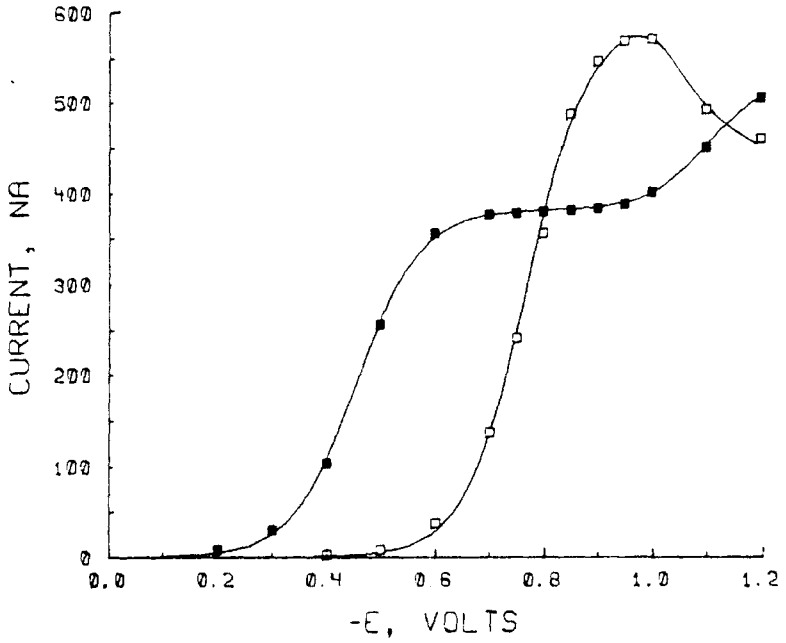

FIGURE 3. Hydrodynamic voltammogram of oxygen on glassy carbon (□) and Au/Hg (■) electrodes. Conditions: 25 x 0.46 cm C_8 Biophase column, 0.1M acetate in 20% (V/V) 1-propanol, 5% ethanol, pH 4.8 at 1 mL/min.

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subpicomole detection of easily reducible substances (e.g. polynitro aromatics, quinones) without any significant interference from dissolved oxygen. The lack of oxygen interference on a GC (E_p is greater than -0.5 V vs Ag/AgCl) simplifies the construction of the reductive LCEC system because it eliminates the need for the oxygen removal apparatus. Even though GC has a smaller negative potential range, it is more reliable and convenient to use than the DME-SMDE or Au/Hg (discussed later in this section) electrode for routine detection of easily reducible analytes.

Other carbon materials such as basal plane pyrolytic graphite (42), and carbon impregnated silicone rubber (43,44) have been used in reductive LCEC with moderate success, but with no readily apparent advantages.

Platinum and gold are the most commonly used solid metal electrodes in electrochemical studies. Application of these electrodes in flow-through transducers have been limited to detection of inorganic ions (35,45-47). They are not attractive for general organic use in aqueous solutions, since formation of oxide layers and adsorption or filming of material at the electrode surface often makes frequent and careful cleaning necessary. These problems are less severe in nonaqueous solvents, where platinum and gold are often an ideal choice.

Since mercury continues to be the material of choice for most electrochemical reductions, several groups have successfully used mercury or amalgamated electrodes on various substrates (e.g. gold, platinum). A very promising improvement in mercury flow-through electrodes has been made by Kissinger's group at Purdue (6,10,21,48) and MacCrehan and coworkers (49,50). They used amalgamated gold thin-layer electordes (Au/Hg) (schematically illustrated in Figure 1B) to prepare a reliable and mechanically rigid transducer for monitoring trace amounts of easily reducible organic and organometallic substances. Such electrodes were also shown to be suitable for detection of substances which react with the amalgam surface to form insoluble salts (51). An Au/Hg electrode has a larger hydrogen overvoltage than GC (but smaller than pure mercury) and thus is better suited for detection of compounds more difficult to reduce. GC and platinum coated with mercury have been used in flow-through detectors (33,52,53) and GC has also been very popular in anodic stripping voltammetry (54). The use of these electrodes in reductive LCEC has not been seriously explored. Other electrode materials (carbides, borides, oxides, nitrides) have been proposed. Semiconductor electrodes have been generally employed in fundamental studies of electron-transfer reactions (55). They do not appear to have any significant advantages over GC or pyrolytic graphite electrodes (22,23).

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A promising electrode material which needs further investigation for reductive LCEC is lead. Lead has been widely used as an reductive electrode material in organic electrosynthesis (56) and its exchange current density for hydrogen evolution is only slightly higher than for mercury.

Approximate negative "potential ranges" for various electrode materials have been summarized (38,39); however, they should be used with caution because they were abstracted from several sources, and the experimental procedures and guidelines for their determination were not uniform. At the present time, GC transducers are most convenient to use at potentials less than -1.0V. Au/Hg are very useful between -0.9 and -1.2 and DME or SMDE electrodes must be used at more negative potentials.

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V. DETECTION MODE

Since the development of polarography by Heyrovsky in the 1920's, the number of electrochemical techniques has been steadily increasing. Over the past eight years, oxidative mode direct current (DC) amperometric detection in liquid chromatography has become widely accepted for solving many problems (trace determinations) of environmental, pharmaceutical, and clinical interest. Recently, several pulse electrochemical techniques have been applied to LCEC detection (41,50,57-63). A debate in the primary literature concerns the relative merits of pulse and DC techniques (in the oxidative mode). This controversy appears to be moving into reductive mode LCEC without serious consideration of the significant differences in the nature of common electrode materials (and ongoing processes at their surfaces) employed in the reductive (mercury base) and oxidative (carbon base) modes of detection. In addition, statements such as "the DC mode of HPLC-reductive amperometric detection was found to be approximately two orders of magnitude

less sensitive than the DP mode" (63) and "As expected, DP mode was found to be approximately 30 times more sensitive than the sample DC mode" (62), tend to confuse the issue without providing any useful information about the detection limits of either technique. It is a well known fact that larger faradaic currents are often associated with an increase in noise that more than offsets the increase in the faradaic current (50,57). Sensitivity, or "response factor" alone does not determine the detection limit (the characteristic of interest). The signal to noise ratio is the critical factor (5,64).

The purpose of this section is to compare the merits of several electrochemical techniques currently being used in reductive mode LCEC and to suggest possible areas which need further improvement and development. The excitation waveforms which have been used in reductive mode LCEC are schematically illustrated in Figure 4.

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DC Amperometric Detection

In DC amperometry the working electrode is poised at a constant potential and the instantaneous current is measured as a function of time (Figure 4A). The magnitude of the current at every point along the voltammetric curve is directly proportional to the bulk concentration of analyte. The selectivity is inversely related to the applied potential.

While it is often desirable to operate the amperometric detector on the limiting current plateau (E_d), in some cases it is advantageous to operate on the rising portion of the voltammetric curve (E_1). Sensitivity is thereby sacrificed for better selectivity. While the sensitivity will be somewhat diminished (to a degree dependent on the exact position of E_1 on the rising portion); in many cases a better signal to noise ratio is achieved due to a correspondingly greater decrease in noise, resulting

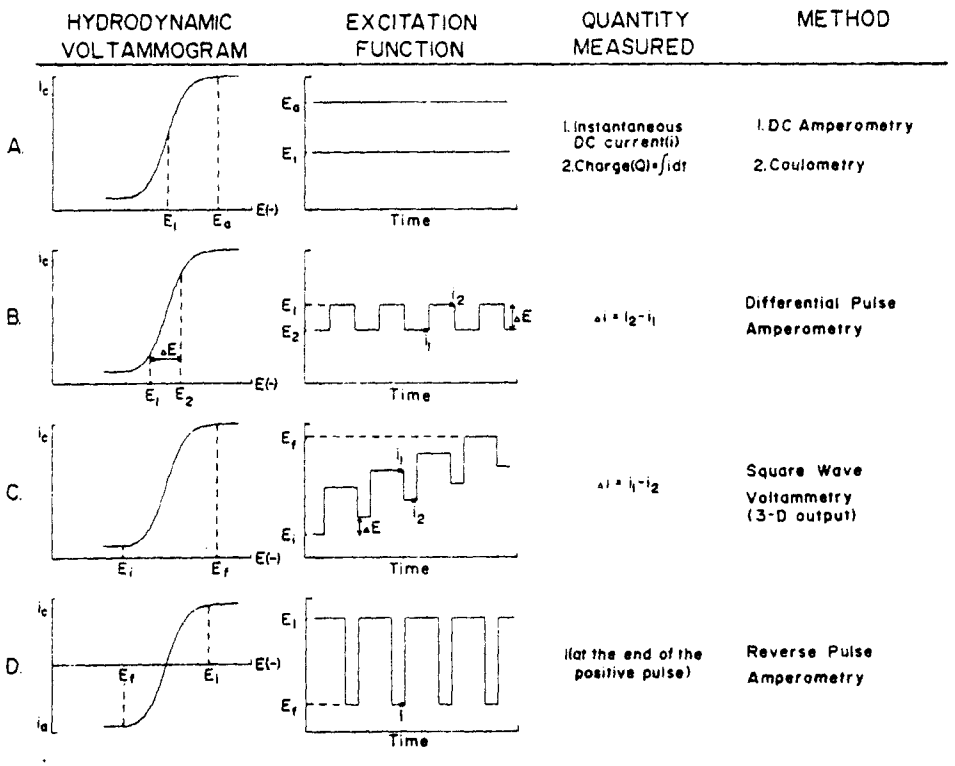

FIGURE 4. Common excitation waveforms used in reductive LCEC detection.

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in better detection limits (39). On the other hand, an amperometric detector operated on the rising portion of the wave is more susceptible to shifts along the potential axis (caused by modifications of the electrode surface, e.g. by adsorption).

The popularity of the DC amperometric detector stems from the fact that the transducer and the associated electronics are simple ir design and offer picomole and subpicomole detection limits at low cost. Typical detection limits for easily reducible molecules are on the order of 1 to 10 picomoles (in many cases detection limits of 0.1 to 1 picomole have been achieved) (10,39,48,65). At the present time, amperometric detection using

thin-layer transducers offers the lowest detection limits because steady-state (constant potential) hydrodynamic electroanalysis has the advantage of being relatively free of double layer charging effects. The major limitations of DC amperometric detection is limited selectivity [e.g. all molecules with reduction (or oxidation) energies lower than or equal to the applied detector potential will be detected].

Differential Pulse Detection

The excitation function used in the differential pulse (DP) amperometric technique is illustrated in Figure 4B. The potential of the working electrode (usually choosen around $E_{1/2}$ of the analyte of interest) is pulsed around a base potential; typically pulse heights are 25-50 mV (ΔE). A larger pulse height can be used to increase the measured current; however, this is achieved at the expense of higher detection limits (the signal to noise ratio follows a bell-shape curve relationship with increasing pulse height) (50). In DP amperometry, the current is measured at both ends of the pulse and the difference in these currents (i_2-i_1)is displayed. The DP current is composed of faradaic and capacitance (charging) currents.

A significant enhancement in selectivity is achieved with the DP mode when compared to the DC amperometric mode. In the DP mode, a compound with a rising portion of its voltammogram outside the window marked by E_1 and E_2 (Figure 4B) will not be detected. In addition, in the DP mode selectivity can be "tuned in" by varying the base potential; a condition which can be successfully exploited for the selective detection of chromatographically separated compounds with similar electrochemically active functional groups. The more selective detection is useful near the "potential limit" of the electrochemical transducer (large negative potentials) and for detection of analytes present

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in very complex samples where the chromatography is inadequate (2,50,57). The baseline drift commonly observed in all detectors (in reductive mode LCEC slow changes in levels of dissolved oxygen and trace metals occur) are minimized or eliminated by the virtue of the differential nature of the output.

Osteryoung and coworkers (66) noted that convection did not effect DP (and normal pulse) faradaic currents as long as the Nernstian diffusion layer is small in comparison to the convective shear layer thickness. Others have shown that this is only true over a narrow range of flowrates and for short pulses (< 200 ms) (57,67). The flowrate independence of DP deemphasizes the need for "pulseless" pumping systems and also because it provides some signal-to-noise improvements (50). Corresponding reduction in S/N is not obtained for "capacitive" noise which is the major limiting noise in DP mode, especially on solid electrodes (2,57). In addition, the DP mode has "put some life" back into mercury pool, DME, and SMDE LC transducers by diminishing the noise effects caused by oscillation of liquid mercury surface in the flowing streams. Differential pulse detection has been recently shown to be successful in minimizing electrode fouling due to adsorption of products of the electrode reaction (50).

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Square Wave Voltammetric Detection and Other Novel Techniques

The excitation function for SW voltammetry is illustrated in Figure 4C. The potential is scanned very quickly (entire scan is completed in 1-2 sec) and the current is measured before and after each pulse step. The forward difference in these currents (i_1-i_2) is displayed either through a simple sample-and-hold circuit or digitally. When coupled to LC, the SW technique provides "simultaneous" multipotential chromatograms which are analogous in many respects to multiple ion monitoring in GC/MS or multiwavelength monitoring in LC/UV (61). Stationary

electrodes can be used to improve the time resolution of current sampling; however, the advantage of a continuously renewable surface is lost. Since high scan rates (100-200 mV/sec) can be achieved, the entire scan can be completed on a fresh mercury drop with SMDE or slowly dropping DME ($t > 400$-500 ms) (61,61).

A very attractive feature of SW detection is its capability of measuring the "peak homogeneity." A three-dimensional plot (in time, potential, and current) would easily reveal the presence of two chromatographically unresolved electroactive analytes. Fouling effects on the electrode surface caused by adsorption of products of analyte reduction are also minimized in SW detection mode.

While the selectivity of SW is better than for DC amperometric mode, detection limits at the present time are one or two orders of magnitude higher. Nonetheless, SW detection is still in its infancy and further refinements will result in lower detection limits. At present, the high cost of SW detectors makes them unattractive for routine applications.

Other newcomers to reductive LCEC detection are reverse pulse amperometry (RP) (68) and dual-electrode thin-layer amperometry (69-71). The excitation function of RP is illustrated in Figure 4D. The current, sampled at the end of the positive pulse, is proportional to the concentration of analytes in the flowing stream. Reverse pulse amperometry appears to have great potential for detection of amalgam soluble metals without interference from dissolved oxygen because E_2 is set at potentials where oxygen is not reduced. However, its application to detection of organic substances is limited to reduction reactions which produce species which are oxidizable (or can undergo a follow-up chemical reaction which generate oxidizable species) and are able to react with the electrode material (e.g. thiol) or adsorb at the electrode surface.

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While most LCEC experiments will continue for some time to be performed with single electrode transducers, it is a relatively simple matter to monitor the current at two different working electrodes simultaneously. This is perfectly analogous to the so-called "dual wavelength UV absorption detector." The electronics needed to accomplish this are straightforward and in principle identical to the circuits used with ring-disk electrodes. The following figure schematically depicts thin-layer transducers with two working electrodes in parallel (A) and in series (B).

The parallel-adjacent arrangement (A) is particularly useful when one wishes to simultaneously quantitate a very easily reduced (or oxidized) substance in the presence of others which react at higher energies. The selectivity for the easily reduced (or oxidized) substance can thus be excellent and other compounds are detected as well. The parallel dual-electrode arrangement can also provide simultaneous measurement of peak current ratios for the purpose of confirming the identity of eluted compounds (just as the absorbance ratio at two wavelengths is characteristic for a given analyte). Both applications save valuable time by permitting a single chromatographic experiment where two would have been required with a single electrode.

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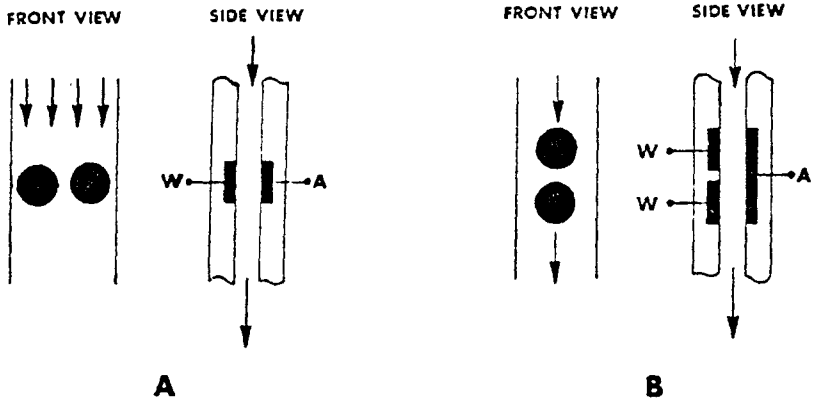

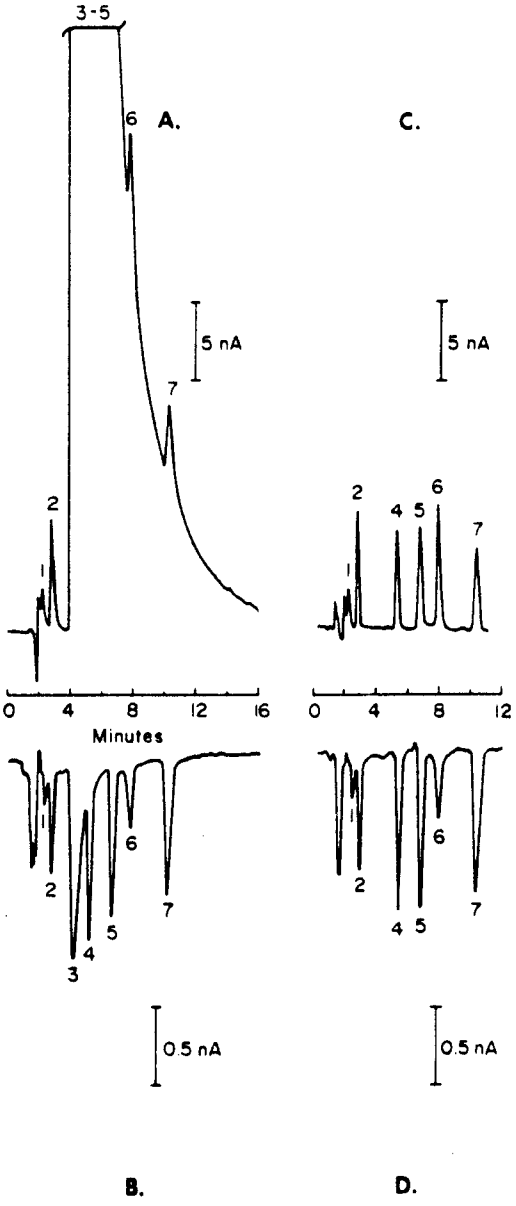

FIGURE 5. Series dual-electrode voltammograms of a synthetic mixture of explosive compounds without (traces A,B) and with (traces C,D) sample deoxygenation. Traces A and C are for the upstream electrode (reductive, E = -0.9V) and Traces B and D

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The series dual-electrode transducer is used in the same manner as the classical ring-disk electrode. The products formed at the upstream electrode are monitored at the downstream electrode. The major application of this scheme is to improve the detection of compounds (e.g. aromatic nitro compounds, quinones etc.) which have reactive products and to enhance selectivity when the electrolysis products can be detected in a more favorable potential region than was necessary to carry out the original reaction. For example, the detection limits for compounds which react at potentials where backgroud processes interfere can be improved by detecting the product(s) in a more favorable part of the available "potential window" (the maximum "potential window" is obtained with a transducer consisting of single GC and Au/Hg electrodes). This is successful because most background (media) reactions are chemically irreversible (e.g. reduction of hydrogen ions, oxidation of water). The series arrangement has another attractive feature, in that it can be used to minimize interference from dissolved oxygen as illustrated in Figure 5. Chromatographically separated sample components along with dissolved oxygen are reduced at the upstream electrode (Figure 5A). The downstream electrode detects compounds which are oxidized at more positive potentials, with minimal oxygen interference (Figure 5B). Oxygen interference at the downstream electrode can be totally eliminated by applying a potential at

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are for the downstream electrode (oxidative, E = +0.8V). The mixture contained 11 picomoles of HMX (1), 5 picomoles of picric acid (2), 18 picomoles of 4-nitrophenol (4), 9 picomoles of TNT (5), 30 picomoles of nitroglycerin (6), and 14 picomoles of 2,4-dinitrotoluene. Peak 3 is oxygen. Conditions: 25 x 0.46 cm Biophase C_{18} column, 0.02M monochloroacetic acid, 0.015 M sodium acetate, 0.001 M EDTA in 17% (V/V) 1-propanol and 5% (V/V) ethanol, pH 3.5, upstream electrode (Au/Hg) was set at -0.90 V and downstream electrode (glassy carbon) was set at +0.80 V, flow rate was 1.7 mL/min.

the downstream electrode which is insufficient to oxidize H_2O_2, which is the product of oxygen reduction. Oxygen must be removed from the mobile phase in order to detect most analytes at either electrode. The full advantage of series dual-electrode (reductive-oxidative) detection approach is achieved in trace determinations where only a limited sample volume is available (< 250 µL) because with oxidative detection at the downstream electrode there is no need to remove dissolved oxygen from the injected solution.

Both the parallel and series transducers provide most of the advantages of differential pulse operation without disadvantages of enhancing double layer charging currents (thus increasing noise) and complicating instrumentation. They extend the specificity and detection limits for thin-layer amperometric detectors and can provide better assurance of peak identity.

In summary, while coupling complex electrochemical techniques (e.g. alternating current, square wave, reverse pulse) to chromatographic separations will provide some fun and challenge for electrochemists and chromatographers in the near future, the workhorses of the reductive (also oxidative) mode LCEC will remain in the hands of single-and-dual-electrode DC and differential pulse amperometry.

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VI. REMOVAL OF DISSOLVED OXYGEN

In every aqueous solution which is in equilibrium with the atmosphere the concentration of dissolved oxygen is on the order of 0.001 M. Dissolved oxygen creates serious problems for reductive mode amperometric detection.

The electrochemistry of oxygen has attracted considerable attention. Oxygen is ultimately reduced to water or hydroxide via a four electron process, however, the electrochemical reduction of oxygen usually proceeds via two well-separated two-

electron steps (72). The first step corresponds to the formation of hydrogen peroxide:

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad \text{Acidic media} \quad (14)$$

$$O_2 + H_2O + 2e^- \rightarrow HO_2^- + OH^- \quad \text{Neutral or basic media} \quad (15)$$

and the second step corresponds to the reduction of peroxide:

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O \quad \text{Acidic media} \quad (16)$$

$$HO_2^- + 2e^- + H_2O \rightarrow 3OH^- \quad \text{Neutral or basic media} \quad (17)$$

The reduction of hydrogen peroxide on glassy carbon and noble electrodes (Pt and Au) in acidic solutions is slow or occurs at potentials more negative than that of the solvent. Oxygen overpotential is much larger on a GC than Au/Hg as illustrated in Figure 3.

A variety of physical methods have been used for removal of dissolved oxygen. The most common method has been sparging with inert gases such as nitrogen, argon, and helium (73,74). Other methods include the use of a nitrogen activated nebulizer (75), refluxing (27,73), vacuum degassing (73), and ultrasonic agitation (73). Nebulizers are not suitable in high resolution chromatographic reverse phase systems because they have large dead-volumes. Even though refluxing of the mobile phase is very effective, bubbles formed at the bottom of the reflux vessel or in the pumping system inlet tubing can enter the LC pump resulting in flow rate fluctuations. Ultrasonic deoxygenation is ineffective, and under certain circumstances it may lead to increased levels of dissolved oxygen. Vacuum deoxygenation and helium sparging were judged to be only partially effective while nitrogen sparging was effective in removal of dissolved oxygen from aqueous solutions (73). Preservation of the mobile phase composition and prevention of the formation of gas bubbles in the detector cell during continuous nitrogen sparging can be

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easily achieved with a reflux apparatus schematically illustrated in Figure 6. It is necessary to maintain continuous nitrogen sparging and heating of the mobile phase (10-15°C above ambient) in order to prevent reentry of oxygen.

A flow-through electrochemical scrubber with a porous silver electrode (20) and an apparatus based on oxygen diffusion through a semipermeable membrane (76) are promising new techniques, but they require more extensive evaluation.

As described above, the removal of oxygen is critical to the operation of a reductive LCEC system. At the same time, dissolved gases, especially oxygen, have a pronounced effect on the performance of UV (below 260 nm) and fluorescence detectors. Changes in concentration of dissolved oxygen cause a UV drift and can decrease the signal in the fluorescence detector (74). A detailed discussion of the role of dissolved gases in liquid chromatography is available elsewhere (74).

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The removal of oxygen from the injected solution can be achieved with nitrogen sparging as illustrated by the schematic diagram in Figure 6. The inlet of the six port rotary valve loop is immersed in sample solution and the deoxygenated sample solution is pulled through the injection loop using a syringe without any exposure to ambient air. With this arrangement, nitrogen is first presaturated with the solvent in which the sample is dissolved in order to minimize solvent loss.

Alternate approaches to oxygen removal methods have been recently proposed by Johnson and coworkers at Iowa State (68), and Kissinger's group at Purdue (70) and MacCrehan (69). Johnson group's approach utilized reverse pulse amperometric detection and Kissinger's group and MacCrehan applied dual-electrode amperometric detection (two electrodes are in "serial" arrangement). Both of these methods detect analytes at potentials where reduction of oxygen and oxidation of hydrogen peroxide does not occur, thus minimizing a major inconvenience of reductive

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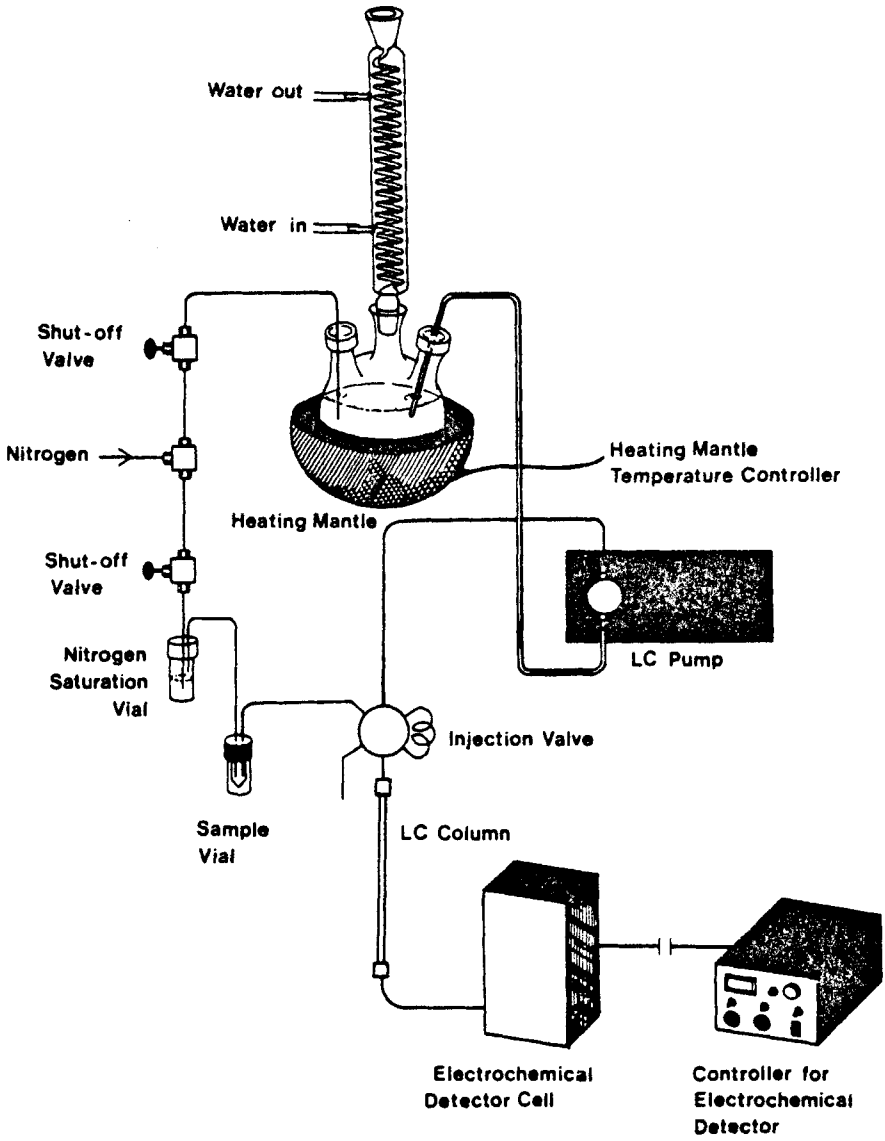

FIGURE 6. Schematic diagram of a reductive mode LCEC system.

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TABLE 1

Recent Applications of Reductive LCEC

Compounds	Tissue or Fluid[a]	Separation Mode[b]	Electrode Material[c]	Detection Mode[d]	Reference
Benzodiazepines	STD/PLA	RP	GC,DME	A,DP	40,50
Benzoylperoxide	STD	RP	Au/Hg	A	79
Cystine	STD	IE	Au/Hg	A	80
Cytrolane, cyolane	WW	RP	Au/Hg	A	6
Diacetol	Feed	RP	DME	DP	41
Diketones, naphthoquinone	STD	RP	DME	SW	60
Dinitrobenzenesulfonyl-amino acids	STD	AD	PG	A	42
Nitramines	EXP	RP	Au/Hg	A	10
Nitrazepam	SER	RP	DME	NP,A	81
Nitroglycerin	GSR	RP	Au/Hg	A	10
Nitrophenol	STD	RP	Au/Hg	A	21
Nitrosamines	STD	RP	DME	NP,DP,SW	60,62,67
Organometals	WW,FDS	RP	Au/Hg	DP	50,82

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Organomercury species	FDS	RP	Au/Hg	DP	49
Parathion	STD,WW	RP	DME,Au/Hg	A	6,83
Pesticides	PLT	NP	DME	A	84
Phylloquinone	PLA	RP	GC	A	85
Testosteroids	STD	RP	DME	A	29
TNT	EXP	RP	Au/Hg	A	10
Ubiquinone	LTH/KTH/PLA/HTH	RP	GC	A	81,86
Vitamin K_3	STD	RP	CP	A	77
Vitamins B_2, B_{12}, K_3	STD	RP	DME	A	83

[a]STD (standard), PLA (plasma), SER (serum), GSR (gunshot residue), EXP (explosive), PLT (plant), WW (waste or runoff water), LTH (liver tissue homogenate), KTH (kidney tissue homogenate), HTH (heart tissue homogenate)

[b]RP (reverse phase), IE (ion exchange), NP (normal phase), AD (adsorption)

[c]Au/Hg (gold amalgam), Ag/Hg (silver amalgam), GC (glassy carbon), CP (carbon paste), DME (dropping mercury electrode), PG (pyrolytic graphite)

[d]A (DC amperometry), DP (Differential pulse amperometry), NP (Normal pulse amperometry), SW (square wave amperometry)

mode LCEC. The theory, limitations, and merits of both of these approaches were briefly discussed in the preceding section of this review.

VII. APPLICATIONS

Many biochemicals, pharmaceuticals, food additives, pesticides, industrial and natural products, and so forth are ideal candidates for reductive LCEC methods development. Derivatives of simple amines, alcohols, and carboxylic acids derived from di-and trinitro aromatic reagents (77) will greatly extend the number of problems which can be attacked by LCEC. Other compounds which react with the mercury surface (e.g. thiols, thioureas) can be selectively detected by mercury-based transducers. Recent applications of reductive mode LCEC are summarized in Table I; however, this list is by no means complete. A frequently updated bibliography of current LCEC applications is available (78).

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