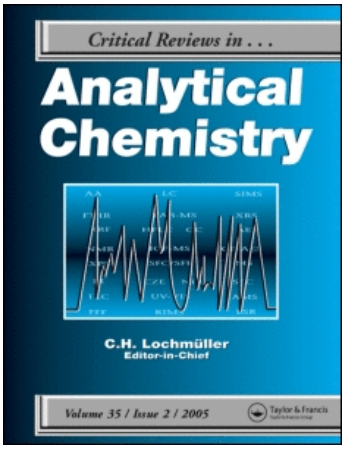

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Electrochemical Detectors in HPLC and Ion Chromatography

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Electrochemical Detectors in HPLC and Ion Chromatography

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I. INTRODUCTION

Back in 1952, the renowned Polish electrochemist Wiktor Kemula introduced chromato-polarography,[1] i.e., polarographic detection for liquid chromatography. This technique continued to develop slowly until the early 1970s (for a review see Reference 2) when modern high-performance liquid chromatography (HPLC) emerged. This new, highly efficient chromatographic method could only be used with detectors ensuring low dispersion. It was not easy to modify the dropping mercury electrode cells to satisfy this requirement. However, at the same time, electroanalytical chemists, who already had much experience in using carbon-based electrodes for oxidative detection in flow analysis, put forward the idea of oxidative amperometric detection in liquid chromatography.[3,4] In this technique, solid or quasi-solid (paste) electrodes were used and this made possible the construction of miniaturized cells with just a few microliter volume.

There was more than that, however, in converting to the thin-layer detector cells. Rather surprisingly, the detection limits of the simple amperometric detector were much lower than 10^{-8} *M*. This observation was made at a time when most electroanalytical chemists were busy packing many modern electronic devices into instruments producing potential waveforms that allowed detection limits of 10^{-7} to 10^{-8} *M* in standard electrochemical cells.

The significant progress achieved in Adams' laboratory was certainly noticed among electroanalytical chemists, but for the wider analytical community, time had not yet come for electrochemical (EC) detection. HPLC was not yet as widely used as it is these days and selective detectors were not so much in demand. It happened, however, that professor Adams and his students were also interested in brain chemistry and they demonstrated the low detection limits of the new device for catecholamines, a group of important neurotransmitters. For some time, brain chemists knew more about LCEC than analytical chemists because they were given exactly what they needed: simple instrumentation, extremely low detection limits, and, most importantly, selectivity toward the substances they wanted to find.

Fortunately, Adams, Kissinger, and co-workers continued their efforts to demonstrate the utility of LCEC for many other analytes like ascorbic acid, uric acid, cysteine, penicillamine, etc. There was also much experience available in the amperometric detection of pharmaceutical agents.[5] More and more electroanalytical chemists began to construct novel EC detector designs: coulometric detectors appeared; the wall-jet geometry was tested in HPLC; experiments were made with potential pulsing, etc. The first commercial detectors came on the market and more and more analytical laboratories ventured into testing this novel, inexpensive tool.

Today, analytical chemists have a wide choice of EC detectors at their disposal. Detection at the static mercury drop electrode has become feasible or, alternatively, mercury/gold film electrodes can be used. Twin working electrodes or larger electrode arrays allow better selectivities or lower detection limits to be achieved. Fast (about 1 s) scanning of a large potential range makes three-dimensional (time, potential, current) detection possible.

Publications dealing with LCEC are rapidly increasing. In 1987, there were well over 200 of them in journals referenced by Chemical Abstracts. Although the proportion of articles dealing mainly with the chromatographic aspects of LCEC are increasing, there is still much activity in detector and electrode development. In the latter respect, the appearance of surface-modified electrodes may become the strongest trend in the future. Such electrodes could bring the tailoring of electrode selectivity within reach and also extend the utility of potentiostatic amperometry to analytes hitherto not detected because of kinetic hindrance of the electrode reaction.

LCEC by amperometry and coulometry has gained so much popularity in recent years that even electroanalytical chemists had to join in the incorrect usage of the term LCEC. There are certainly other types of LC detectors that have a right to bear the name electrochemical: potentiometric, conductometric, oscillometric, tensammetric, and permittivity detectors continue to be used. Since ion chromatography became so popular, there are probably more conductivity detectors in use than amperometric. The other EC detectors are much less frequently used. In this review, we use the term LCEC both in the broader sense, meaning all detection principles, and in the popular way for amperometric, coulometric, and polarographic detection. The latter three methods are also referred to as voltammetric detection, which would probably be the correct alternative of EC when only these methods are meant. We restrict the term polarographic to the dropping mercury electrode.

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A. Scope of this Review

With well over 200 related publications a year, LCEC has become yet another of those fields where an individual may not be able to follow all new developments.

Fortunately, the number of review articles and book chapters is also mushrooming. One of the most recent outcomes has been the book *Electrochemical Detection in Medicine and Chemistry*[6] consisting of 16 individual reviews or articles. The book has been reviewed by Frei.[7] Another recent publication is Stulik and Pacakova's *Electroanalytical Measurements in Flowing Liquids*.[8] The authors have written many reviews on the subject in the past and have also been actively involved in detector and method development. A large part of the book is devoted to practical applications with emphasis on LC. This part alone enumerates 368 references. Shoup, one of the most experienced researchers involved in LCEC progress, has authored a 104 page chapter on LCEC.[9] Many other, shorter reviews have also been recently published. Kissinger[10] wrote on fundamentals and future directions of LCEC, and Sharp[11] reviewed theoretical aspects. Horvai and Pungor [12] and Frischkorn et al.[13] have discussed recent trends. Criteria for choosing the right working electrode material have been presented by Rocklin[14] and polarographic techniques for flow analysis were compared by Kubiak.[15] Leroy and Nicolas[16] wrote on derivatization for LCEC (in French) and Smyth et al.[17] reviewed LCEC of environmentally important organic compounds. Two other reviews,[18,19] although of wider scope, are also relevant. Last, but not least, an excellent review was written[20] by six outstanding experts of LCEC on electroanalytical voltammetry in flowing solutions.

The present review includes a primer of EC principles of voltammetric detection for those readers who are less familiar with electrochemistry. The main part of the review presents LCEC applications. Our purpose was to concentrate on recent developments in the traditional fields of application and to draw attention to those areas where research has been very active recently. Finally, the progress in instrumentation is briefly scanned.[6]

Our choice of weighting the material was clearly influenced by the fact that an excellent review on LCEC was published in this journal,[21] which more extensively dealt with theory and detector cells but gave only a brief sketch of the applications. Smyth's review,[19] also in this journal, treated in detail the electroanalytical chemistry of the most important classes of analytes in LCEC.

II. ELECTROCHEMICAL BACKGROUNDS

Conductivity and permittivity detectors measure bulk properties of the column effluent. In contradistinction, potentiometric detectors measure electromotive force (emf), i.e., the potential at the surface in equilibrium with the contacting solution. Because many potentiometric sensors show high selectivity, detection often can be selective for a particular ion.

The principles underlying the measurement of conductivity, permittivity (dielectric constant), and emf are widely known and need no treatment here.

A. Amperometry

Amperometry is a special way of performing electrolysis in a sample solution. Figure 1 shows the scheme of a simple electrolysis cell. The DC voltage applied to the cell generates current I.

Since electrical charge is carried by ions in the sample solution and by electrons in the electrodes and wiring, there is a change of charge carrier type at the electrode/electrolyte interface. In other words, electrons arriving from the metal to the interface have to be picked up by ions or molecules. These ions or molecules will be consequently reduced. The electrode where this occurs is called the cathode. At the other electrode, the anode, electrons enter the metal and some chemical species is oxidized.

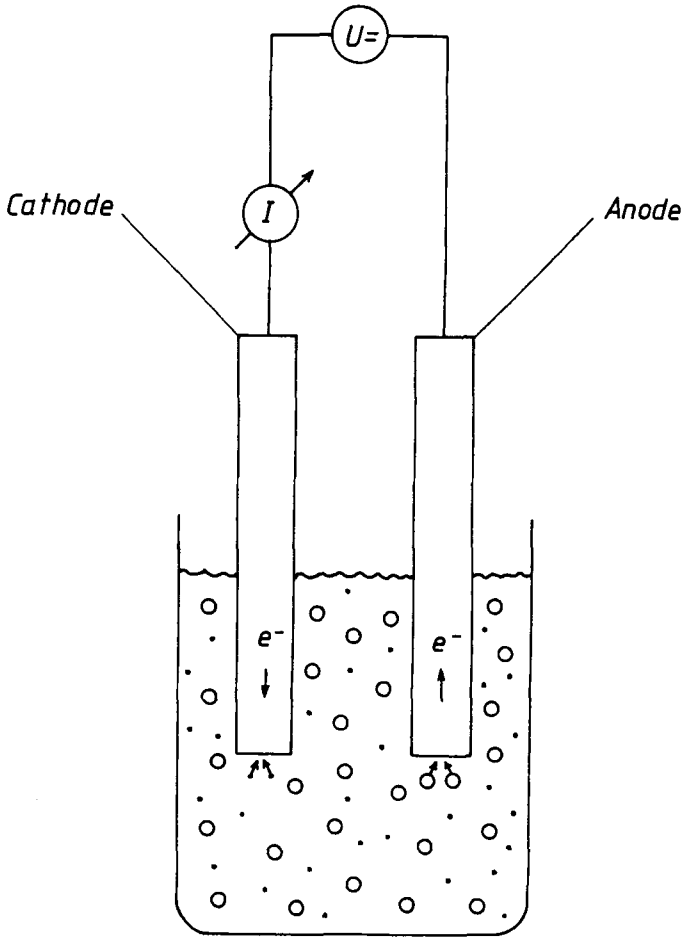


FIGURE 1. Simple electrolysis cell. Two electrodes are immersed in an electrolyte solution. A DC voltage, U, is applied across the electrodes. Current I, is measured by an ammeter. Reducible species in the solution (e.g., Fe(III), chinon) diffuse to the cathode to pick up electrons. Oxidizable species (e.g., Fe(II), hydrochinon) diffuse to the anode to give up electrons there, i.e, to be oxidized.

Current intensity is expressed by the number of charges or number of electrons passing at a cross section of the leads in 1 s. One such cross section is the interface electrode/electrolyte where charge transfer occurs via oxidation or reduction. It follows that the current intensity and the rate of charge transfer at the interface are equivalent and their numerical values are directly proportional.

As a result, the relationship between the voltage applied and the current generated is not a linear function as in Ohm's law. Instead, it depends on a number of factors which affect the rate of the surface reaction, such as the composition of sample solution, electrode material, and rate of mass transfer in the sample solution. In typical cells, the ohmic resistance of the circuit is negligible, and the relationship between current and potential is determined by the charge transfer and/or diffusion processes at the two electrode/electrolyte interfaces.

B. Three-Electrode Cells

The situation is simpler when one has to consider only one electrode/electrolyte interface at a time. Paradoxically, this can be done by using three electrodes as shown in Figure 2, which illustrates a typical scheme employed in amperometric detectors.

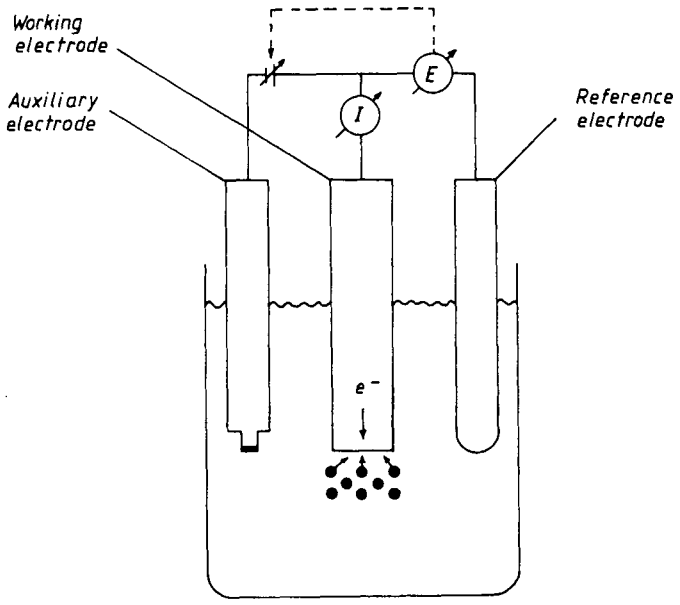


FIGURE 2. Electrolysis with a three-electrode cell and potentiostatic control. This cell is used to obtain information from a single electrode/electrolyte interface, i.e., between the working electrode and the sample. Current, I, passing through the working electrode returns through the auxiliary electrode. The potential, E, of the working electrode is measured against the currentless reference electrode. The DC voltage source is controlled to keep E at a preset value.

What is measured is the rate of electron transfer at the interface between the working electrode and the sample solution. This rate is a function of the applied voltage and of the concentration of the components participating in the reaction. The current generated by the DC voltage source passes through the auxiliary electrode into the electrolyte and returns through the working electrode. The potential of the working electrode is measured against the current-free reference electrode by a high input impedance amplifer. In order to ensure exact conditions for the measurement, either the current or the potential should be kept constant by means of a control loop.

In amperometry, the potential is kept constant and the current is measured, hence the name potentiostatic amperometry.

C. Detector Cell Geometries

The design of practical amperometric detector cells differs only in the cell geometry from that shown by the schematic in Figure 2. The most important feature, of course, is the low dead volume which avoids undue band broadening. In a thin-layer or sandwich-type cell,[3] such as the one shown in Figure 3, the effective cell volume is defined by the elongated cavity formed by the thin spacer.

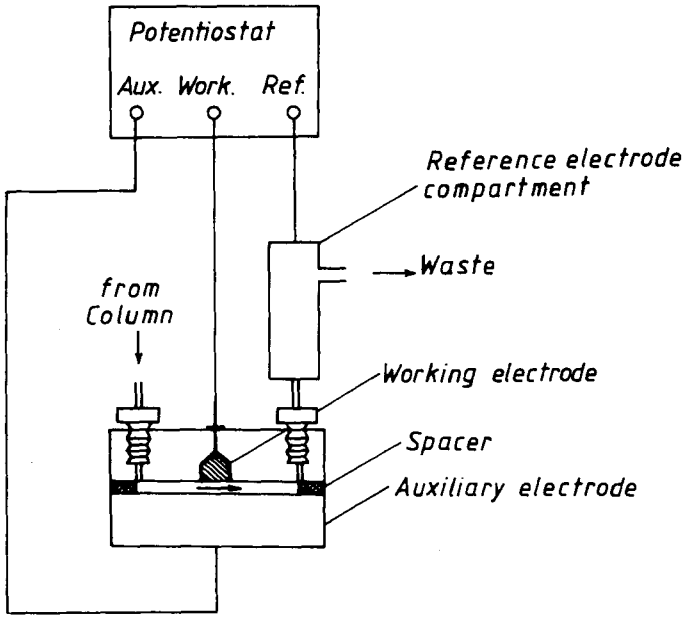


FIGURE 3. Detector with thin-layer cell. The column effluent passes through a narrow (50 to 100 μm) space between the stainless steel auxiliary electrode block and the plastic, e.g., KEL-F block. The working electrode, e.g., a 4-mm diameter glassy carbon rod, is fixed in the cavity of the plastic block. The blocks are about 25 × 25 × 10 mm.

On the other hand, in the wall-jet-type cell of Figure 4, the total cell volume may be more than 10 ml, yet the effective volume calculated from band broadening in the detector is less than 10 μl.[22-24]

Other cell geometries, such as tubular, annular, or porous, also have been used but are less popular than thin-layer or wall-jet cells.

In amperometry, the flow rate of the liquid has to be maintained constant in order to ensure a constant rate of mass

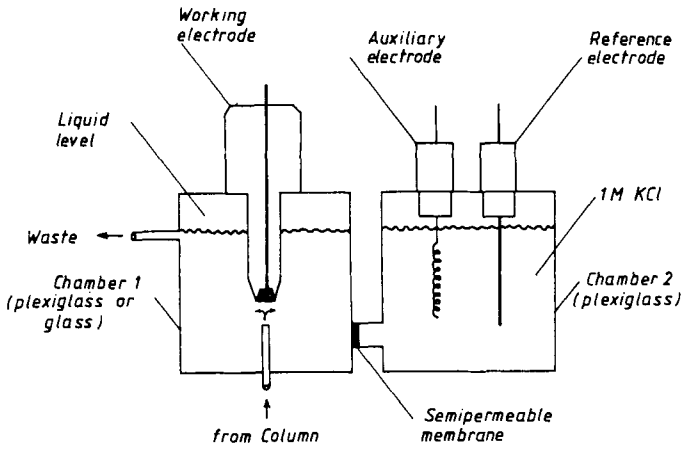


FIGURE 4. Wall-jet-type cell. The column effluent leaving the capillary tubing forms a liquid jet and spreads into a sheath on the surface of the working electrode. The latter responds only to this streaming film and does not sense the large, ca. 15 ml, volume of liquid in chamber 1.

transfer to the electrode surface that is the site of the redox reaction.

As noted earlier, cell resistance usually is quite low due to the presence of electrolytes in the eluent. Most detectors cannot perform with solutions of high specific resistance, e.g., eluents rich in organic solvents. Large-volume wall-jet cells such as that shown in Figure 4 have been said to overcome this difficulty.[25]

D. Electrode Materials

The most frequently used working-electrode materials are different carbons, e.g., glassy carbon, pyrolytic carbon, or carbon paste, or metals such as gold, platinium, or silver. These materials are used mainly with oxidizable analytes.

On the other hand, mercury and amalgams are most often used when the analyte has to be reduced at the working electrode.

Carbon-based electrodes have been much more frequently used than the other electrode materials. Glassy carbon, which is a special product, is available only from a handful of manufacturers. Glassy carbon electrodes are most frequently used because they are rugged and resist organic solvents. However, they need very careful surface preparation to achieve low detection limits and have to be slightly repolished from time to time depending on usage. In addition, excessively high currents may cause irreparable damage to the electrode.

Carbon pastes are prepared from pure graphite powder with liquids such as paraffin oil or silicone oil. Detection limits are excellent, but the life span of each electrode is relatively short, i.e., a few weeks or months, and reproducibility between paste preparations is difficult. Nevertheless, spoiled electrodes can be rejuvenated with fresh carbon paste.

In contrast to glassy carbon, carbon pastes cannot be used in contact with eluents containing high concentrations (e.g., above 25%) of organic solvent, e.g., methanol or acetonitrile. However, promising results have been obtained in reducing this problem by using silicone rubber embedding material[26] and polyethylene[26a] in preparation of the carbon pastes for electrodes used in LC.

E. Polarization Voltage

The potential of the working electrode vs. the reference electrode is adjusted according to the particular use of the electrochemical detector, i.e., the polarization voltage is chosen by considering the required selectivity and signal-to-noise ratio.

As an example, let us consider the oxidation of an analyte at a glassy carbon working electrode. Figure 5 shows that the current passing through the working electrode depends on both the applied polarization voltage and the composition of the solution flowing through the detector cell.

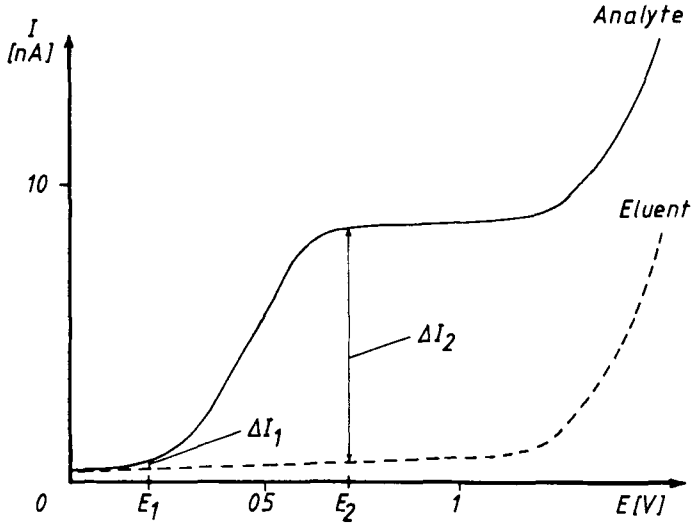


FIGURE 5. Current-voltage curves of analyte and eluent. If the polarization voltage is set to E_1, the useful part of the signal is ΔI_1, whereas at E_2 it is ΔI_2.

Unless a rather high positive potential is chosen, the eluent does not react significantly at the electrode and therefore does not contribute significantly to the current. The analyte, however, gives rise to a detactable current at any potential greater than E_1.

The current increases with the potential between E_1 and E_2, but afterwards it reaches a plateau. The height of this plateau, i.e., the current at and above E_2, is directly proportional to the concentration of the analyte. This proportionality usually extends over 3 to 5 orders of magnitude, so that the linear dynamic range of the amperometric detector is quite wide.

The values of the two threshold voltages E_1 and E_2 vary from analyte to analyte. The more easily the analyte is oxidized, the lower E_1 and E_2 will be.

For any given analyte, the optimum polarization voltage is given by the value of E_2, where the useful signal reaches its highest attainable value. Although the signal remains practically

constant upon increasing the potential above E_2, the background signal due to the eluent will increase. Since this increase contributes noise to the useful signal, the lowest detection limit usually is obtained at E_2. Detecting at a potential on the flat plateau of the I-E relationship is also favored because of the insensitivity to small changes in detector potential.

In chromatography, different analytes are detected one after another. Since it is not convenient to change the polarization voltage during a chromatographic run (because of large current transients following the voltage jump), the level selected is a choice between detecting more compounds with less noise.

Many organic compounds are not oxidizable in a potential range where the background current is sufficiently low. Only those containing readily oxidizable functional groups are detectable by the amperometric detector, and this is the basis of its selectivity.

Readers interested in more detail about the EC backgrounds of amperometric detection are recommended to peruse a book[27] edited by Kissinger and Heinemann and two reviews.[9,20]

F. Coulometric Cells

During the passage of the sample through an amperometric detector, a small fraction of the analyte is lost, i.e., oxidized or reduced at the surface of the working electrode. On the other hand, nearly 100% of the analyte is reduced or oxidized in coulometric cells where the effective surface area of the working electrode is much greater. The coulometric detector, which may have thin-layer or porous flow-through geometry, is a particular kind of amperometric cell.

The current passing through the coulometric detector's working electrode is much higher than that in a given amperometric detector. There is also a concomitant increase in noise, however, with no improvement in the detection limit or the signal-to-noise ratio. Variations of flow rate do not affect the analyte signal in coulometric detectors because there is no way to increase conversion above 100%.

G. Purity of Reagents and Water

The high sensitivity of electrochemical detectors makes it feasible to analyze biologically active substances with low detection limits. Take for example the detection of 200 pg of a substance with molecular weight 200. The peak should be eluted in 12 s at a flow rate of 1 ml/min. The concentration of analyte in the detector at the peak maximum is then approximately 10^{-8} *M*.

The eluent may contain electroactive impurities at this or even higher levels if sufficient care is not taken. Electroactive impurities may also be leached out from certain parts of the chromatographic system and/or introduced with the eluent or the sample.

H. Controling Baseline Noise

A set of useful hints has been given by one manufacturer[28] of LCEC detectors for troubleshooting when the baseline is noisy. It may be helpful to consider this checklist also with other detectors: (1) air bubbles in the system; (2) electrical grounding not correct; (3) microscopic leaks present; (4) reference electrode potential not right or junction of reference electrode clogged; (5) mobile phase needs degassing or replacement; (6) background current unusual; (7) inspection of the working electrode; (8) pump seals worn and leaky; (9) check valves need sonicating; (10) stainless steel parts need passivation; (11) check the electronics of the potentiostat.

III. BIOGENIC AMINES[29-36]

Successful detection of biogenic amines separated by LC has played the key role in the development of EC detection and it appears to be still the most frequently quoted field of application for LCEC. Biogenic amines are aromatic amines produced by living organisms. They are usually found only in minor concentrations, but they are of vital importance, particularly in the chemical information system of the organism (neuroregulators).

Two families of biochemically interrelated compounds have been investigated the most: the catecholamines with their metabolites, and the tryptophan derivatives. Figures 6 and 7 show

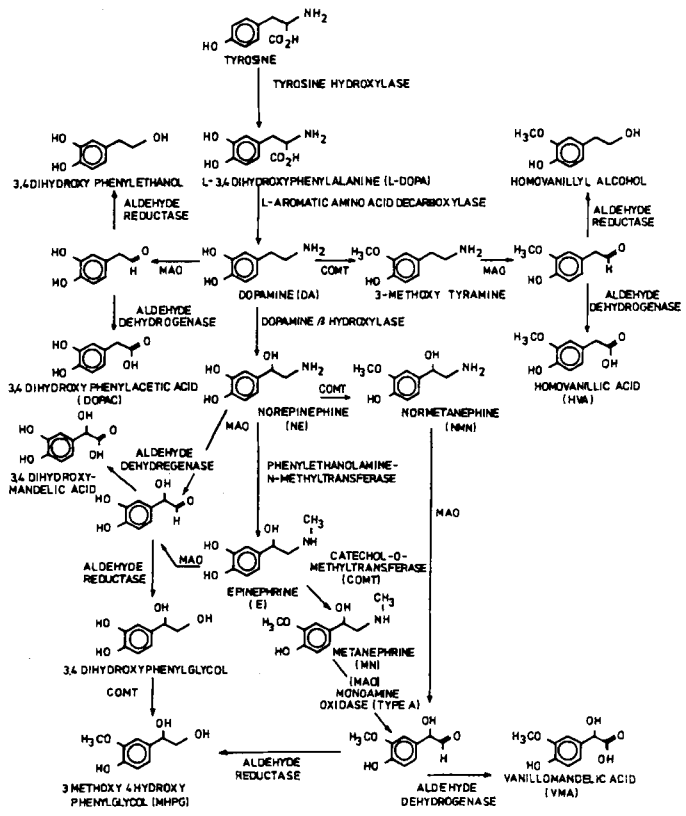


FIGURE 6. Metabolic pathways for synthesis and degradation of catecholamines and related compounds. (From Mefford, I. N., *Methods Biochem. Anal.*, 31, 221, 1985. With permission.)

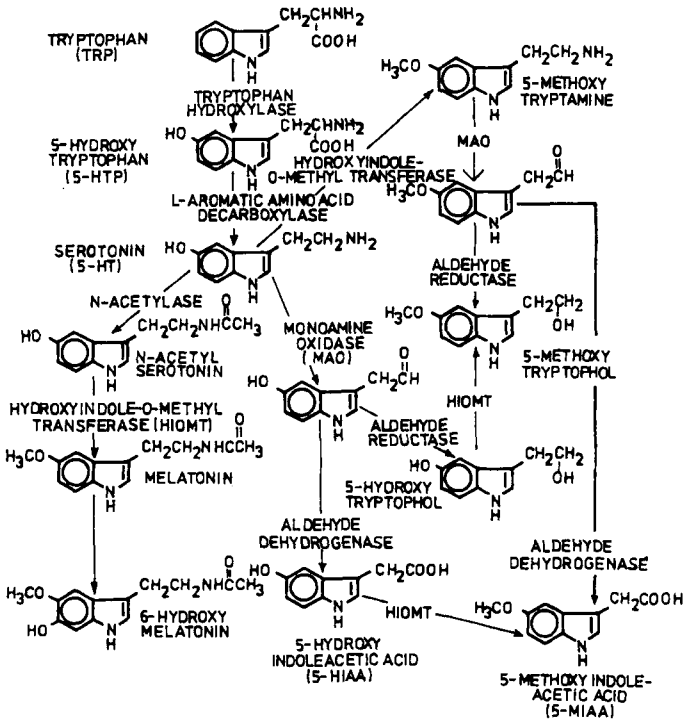


FIGURE 7. Metabolic pathways for synthesis and degradation of tryptophan metabolites. (From Mefford, I. N., *Methods Biochem. Anal.*, 31, 221, 1985. With permission.)

the structures of compounds belonging to each group and the enzymatic pathways between them. Tyrosine and tryptophan are the respective parent molecules in the two families. The term catecholamine relates to molecules containing a 1,2-dihydroxyphenyl unit attached to an aminoethyl group (dopamine, epinephrine, norepinephrine). "Biogenic amines" is a loosely defined term, it is mostly used to refer to the most important representatives of the neuroregulatory compounds, the catecholamines and 5-hydroxytryptamine (serotonin),[29] but it is often understood to imply also their precursors and metabolites. The latter meaning is used in this review. The names of the compounds shown in Figures 6 and 7 are often quoted by abbreviated names. These are shown in the figures and a selection is also given in Table 1 for easy reference.

Workers using LCEC to determine biogenic amine concentrations have been mostly involved in either of two large fields: research into neuroregulation or investigation of the clinical usefulness of quantitating neurotransmitters and their degradation products in body fluids. Recent years have seen tremendous activity in neuroscience. This can be partly attributed to the availability of analytical tools suitable for studying very low concentrations or miniscule quantities of neuroregulatory compounds. Of course, these analytical methods have not been limited to LCEC. Non-EC techniques like fluorescence, radioassay, mass spectrometry, and others have been frequently used. On the other hand, EC sensors have also been used *in vivo* to measure neurotransmitters in localized brain areas of

Table 1
Frequently Used Abbreviations of Some Neurotransmitters and Metabolites

DOPA	3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenylacetic acid
DA	Dopamine
E	Epinephrine
NE	Norepinephrine
MN	Metanephrine
NMN	Normetanephrine
HVA	Homovanillic acid
VMA	Vanillomandelic acid
MHPG	3-Methoxy-4-hydroxyphenylethyleneglycol
5-HT	5-Hydroxytryptamine or serotonin
5-HIAA	5-hydroxyindoleacetic acid

experimental animals. As a result of these efforts, many of the compounds in Figures 6 and 7 could be shown to be involved in a number of psychological and physiological functions, including locomotion, hunger, thirst, sleep, and sexual activity. Neurological and psychiatric disorders, e.g., migraine, schizophrenia, depression, anxiety, pheochromocytoma, and parkinsonism, appear to be related to neurotransmitter metabolism. Psychotropic and cardiac drugs are thought to interact with catecholamine metabolism and/or receptor activity.

Typical samples for chemical analysis in this field are brain tissue, aqueous solutions used to extract analytes from the brain *in vivo* (microfiber dialysis, push-pull cannula), cerebrospinal fluid (CSF), blood or serum, and urine. Except for urine, the amount of sample is small and the analyte concentration is low so that picogram to nanogram quantities of the analyte have to be detected and quantitated. This means that the analytical techniques are frequently used near their detection limits. Sampling artifacts are also not rare. The conclusion is that results have to be validated rather carefully.

Another consequence of working near the limits of the capability of the instruments is that in the analysis of the biogenic amines and related compounds by LCEC the problems of chromatographic separation and detection have to be dealt with simultaneously. It is appropriate therefore to discuss the HPLC techniques used in biogenic amine analysis.

A. HPLC Separation of Biogenic Amines

The biological samples analyzed for biogenic amines are too complex to allow full separation of all constituents by LC alone. They may also contain components such as proteins, which slowly deteriorate column performance. Suitably chosen sample pretreatment can help in two ways: it removes harmful substances and it reduces the number of noninteresting components. A further way to simplify the separation is to use selective detectors like the fluorescence or the EC detector. Many peaks that would interfere if a universal detector were used are not "seen" by these detectors. Amperometric and coulometric detectors used in the oxidative mode are selective

for the easily oxidized catechol-, phenol-, and indole derivatives which constitute the majority of biogenic amines.

When a suitable pretreatment and a selective detector are used, the remaining separation problems can be dealt with by the LC methodology. Normal phase separations have rarely been used to separate biogenic amines, apparently because normal phase sorbents are too easily deteriorated by biological samples. Ion exchange chromatography was extensively used for biogenic amine separations, particularly in the 1970s when reversed phase materials were still in development. For ion exchange to occur, the analyte has to be present in ionized form. In sufficiently acidic buffers, the amines are protonated to cations, while organic acids can be deprotonated to anions in buffers of near neutral or basic pH. Therefore, amines can be chromatographed on cation exchangers and acids on anion exchangers. Neutral metabolites of the biogenic amines, e.g., MHPG, are not retained, however, on ion exchangers.

Reversed phase sorbents have now almost completely replaced ion exchangers in biogenic amine analysis. These packing materials are not limited to ionizable analytes and provide much more versatility than ion exchangers. In ion exchange, elution order can only moderately be influenced, whereas in reversed phase work the experimenter has a number of parameters at hand for influencing retention. These include mobile phase pH to control analyte ionization; organic modifiers to change mobile phase polarity and interaction with the sorbent; and ion pairing agents to reduce analyte polarity. There are several choices for the organic modifier (methanol and acetonitrile have been used most frequently) and a variety of ion pairing agents with individual effects is available, e.g., sodium laurylsulfate, sodium octanesulfonate, etc. Finally, there is a choice of reversed phase materials depending on the chain length of the covalently bonded hydrocarbon groups or only in the production technology of the stationary phase which may in itself cause considerable differences in separation. Reversed phase materials are more versatile to effect many different separations and they are also suitable for the simultaneous analysis of an ionizable parent compound and its nonionizable metabolite. Plate counts on reversed phase materials are generally better than on comparable ion exchange columns. On the other hand, separation based on ion pairing requires more operator care and experience, long equilibration times, and adjustment of the mobile phase when the column is aging. For these reasons, it is less suitable for clinical routine analyses and for automation.

B. Detection of Biogenic Amines

Biogenic amines have been detected mostly by UV, fluorescence, and EC detectors. Since LC equipment nearly always includes a UV detector, this would be the first choice for a detector. The biogenic amines are aromatic compounds with reasonably high UV absorption. Detection limits are in the low microgram range. This is not enough for blood catecholamines but is sufficient for urinary VMA and HVA. Another factor limiting the usefulness of UV detection is that the UV detector is not sufficiently selective: there are too many UV-absorbing species in biological samples and chromatographic interferences are almost unavoidable.

Fluorescence detection has lower detection limits than UV, somewhere in the low picogram range, and there are only very few interferences because only a few biogenic materials fluoresce. The native fluorescence of some catechols and indoles, e.g. indoleacetic acid, is sufficient for sensitive detection, but most of them need to be derivatized, pre- or postcolumn, with fluorogenic compounds or by oxidation to fluorescent tryhydroxyindoles. Of course, derivatization makes the detection scheme more involved. Automated derivatization makes the technique more convenient but also more costly. Fluorescence is also subject to quenching and this limits the choice of eluent compositions. Finally, there are biogenic amine derivatives which cannot be derivatized with known reagents to fluorescent compounds. In spite of these limitations, fluorescence detection has been widely used in biogenic amine detection to solve specific problems.

Voltammetric EC detectors have become widely known and used only after they had conquered the field of biogenic amine analysis. This is certainly due to their particular suitability for this purpose. Their sensitivity allows detection of picogram quantities of many neurotransmitters, and their selectivity for easily oxidizable substances greatly reduces the possibilities of interference. Since catechols and hydroxy indoles are oxidized at lower positive potential than phenolic and indole compounds, the choice of working potential or the use of dual sensors may add a further dimension of selectivity. The role of EC detectors in biogenic amine analysis is discussed in more detail later. Suffice it here to say that there are also drawbacks of this technique, not the least of which is the possibility of electrode fouling.

C. Sample Preparation

Sample preparation depends greatly on the separation provided by the column and on the selectivity of the detector.

With certain samples only a minimum of pretreatment is necessary. For example, brain samples are often analyzed after homogenization and removal of proteins and cell debris by centrifugation. Such direct injection assays can have improved sensitivity and reproducibility. However, most published HPLC methods use more extensive sample prepurification. This often leads to considerable loss of analyte and thus less sensitivity and higher variance. To minimize such effects, internal standards are added to the sample before pretreatment. If the internal standard is sufficiently similar to the analyte, then inaccuracies due to sample pretreatment loss and to change in detector sensitivity can both be corrected for.

Three clean-up procedures are commonly used: ion-exchange separation, selective adsorption, and liquid-liquid extraction. Typically, the analyte(s) are adsorbed or extracted from the pH-adjusted sample. Subsequently, the analyte is recovered, possibly into a smaller volume of buffer or acid solution than

the original sample. In this way, the analyte is not only purified but also concentrated. Ion exchangers and other sorbents are most often used in easily handled small bed, disposable columns. The purification of catechol derivatives is based on the selective interaction with alumina or borates.

D. Voltammetric Detection of Biogenic Amines

Many biogenic amines and related compounds are easily oxidized at a carbon electrode. Typical oxidation reactions are shown in Figure 8.

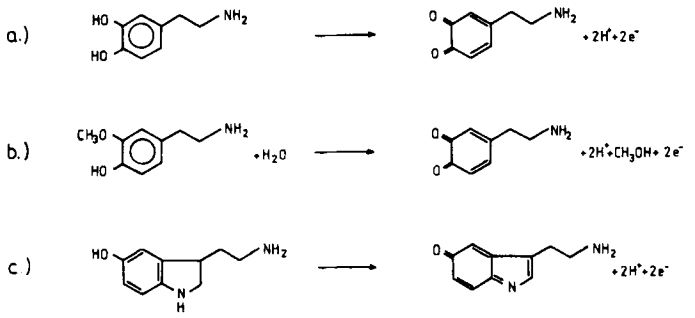


FIGURE 8. Typical oxidation reactions of (a) catecholamines, (b) O-methylated catecholamines, and (c) 5-hydroxyindoles.

Catechol derivatives are oxidized at carbon electrodes at approximately 0.2 V vs. SCE at physiological pH, hydroxyindoles at slightly higher potential, methoxylated catechol derivatives like HVA near 0.4 V and tyrosine at about 0.6 V vs. SCE. At lower pH, the oxidation potentials are higher. Frequently encountered interferants are ascorbic acid and uric acid, themselves easily oxidized. Most other substances found in brain and body fluids are not electroactive at these potentials.

The inherent shortcomings of bulk solution electrochemistry are mostly circumvented when low concentrations of biogenic amines are measured by LCEC. Working at constant potential eliminates charging currents. Electrode filming is virtually absent due to extremely low analyte concentrations and constant washing of the working electrode's surface by eluent. Also, strongly adsorbing species in the sample stick to the column and never reach the detector. Irreversible electrode reactions may reduce the sensitivity but do not disturb otherwise. All these factors make the use of EC detectors feasible in biogenic amine detection in biological fluids. Yet the efforts for improved selectivity and still lower detection limits have not abated. Caliguri and Mefford[38] have been advocating the use of microbore HPLC columns of 1.1-mm I.D. with 3 micron packing. Samples undergo less dilution when passing a microbore column than in conventionally sized columns. Detection limits in the femtogram range can be thus achieved. Employing novel surfactants to modify the stationary phase, the same author could also enhance the selectivity.[39] Figure 9 shows plasma and CSF analyses with the microbore system.

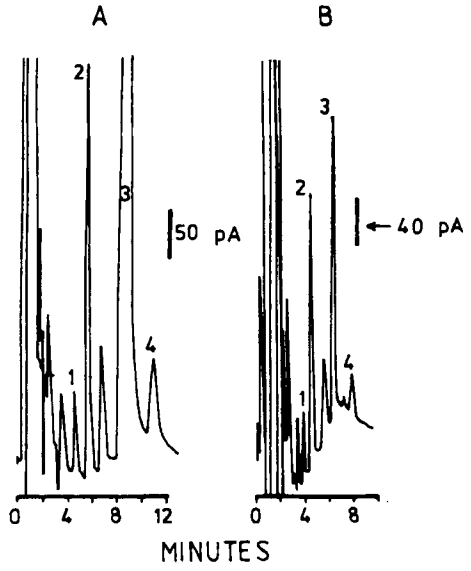


FIGURE 9. Microbore resolution of 40-μl alumina extracts of plasma (A) and cerebrospinal fluid catecholamines (B). Peak identities: (1) epinephrine, (2) norepinephrine, (3) dihydroxybenzylamine (internal standard), and (4) dopamine. (From Mefford, I. N., *Life Sci.*, 41, 893, 1987. With permission.)

Selectivity can be improved also by electrochemical means. Dual or, in general, multiple electrode detectors may add a further dimension of selectivity either in the parallel or in the series configuration.[40] As an example, the interference in brain NE measurement due to MHPG could be eliminated in this way (Figure 10). NE oxidized at the upstream electrode could be reduced and thereby quantitated at the downstream electrode. The interfering MHPG was irreversibly oxidized at the upstream electrode and gave no signal at the downstream electrode. A series dual coulometric cell was used to increase the selectivity in the following manner.[41] Easily oxidized interferants were almost quantitatively oxidized at the upstream electrode, set at +0.22 V. The downstream electrode was set at +0.42 V and recorded the harder-to-oxidize methoxylated metabolites HVA, MHPG, and VMA. This work also demonstrated that gradient elution is feasible in the LCEC determination of tyrosine metabolites.

A series array of 16 coulometric electrodes has been used in a similar fashion.[42,43] The members of the series were set at incrementally increasing potentials. Compounds oxidizing at the first electrode are completely converted so that they do not reach the second electrode, those oxidizing at the second electrode do not arrive at the third electrode, etc. Thus, each electrode oxidizes — and thereby detects — only those compounds which were not oxidized at the next lower potential but can be oxidized at the potential of this electrode. Conceptually, one can obtain 16 simultaneous, independent chromatograms, where each peak is described not only by elution time, but also by the potential of the electrode where

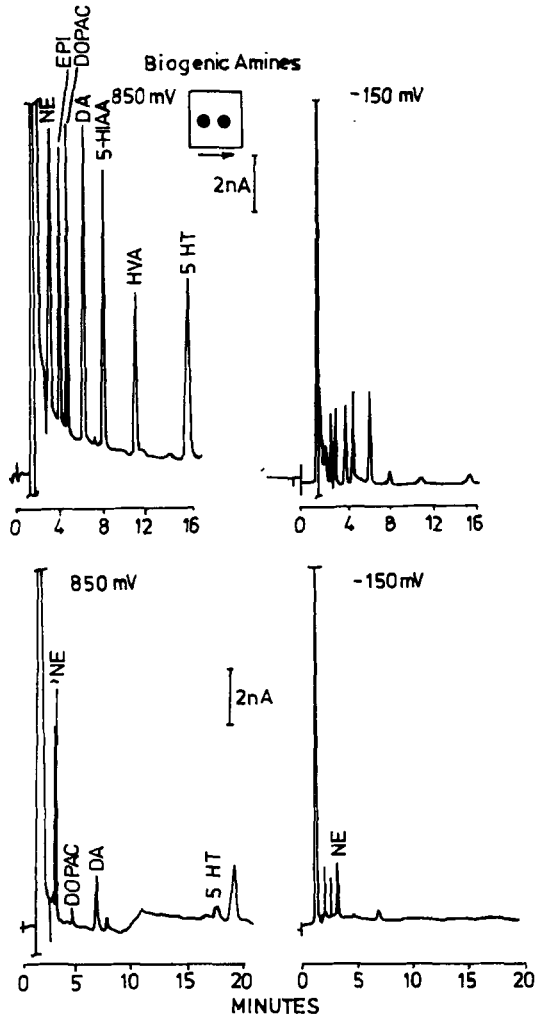


FIGURE 10. Simultaneous series dual-electrode chromatograms of rat brain tissue homogenate. The peak immediately preceding NE at the 850-mV electrode is MHPG, which is subsequently filtered out at the downstream electrode at −150 mV. (From Mayer, G. S. and Shoup, R. E., *J. Chromatogr.*, 255, 533, 1983. With permission)

it was detected. In practice, oxidation of analytes cannot be strictly localized to a single electrode, and leading and following electrodes are reported to have a typical 10% share in oxidation. Potential separation of neighboring electrodes could be brought down to 2 to 5 mV and bandspreading at the 16th sensor was only 30 to 40 μl. The fully nonmetallic system was compatible with gradient elution. The authors report that directly injected biological fluids, e.g., CSF, saliva, urine, brain or plasma extracts, typically yielded 80 to 120 resolved isocratic peaks and up to 300 gradient peaks.

Dual electrodes in the parallel-opposed configuration have been used to increase sensitivity by the amplification due to oxidation/reduction cycles in this detection scheme.[44] Detection limits of 3 pg for NE and 5 pg for DA have been achieved in this way.

Although the usefulness of more sophisticated EC detection schemes has been demonstrated by examples like those quoted, the majority of researchers still appears to work with the single working electrode cells. The simplicity of this detector type will probably remain appealing to many clinical and other routine laboratories. To make efficient use of these instruments in the hands of medical technologists, a number of precautions should be obeyed[45] (Table 2).

Table 2
Precautions for Reproducible Analyses with Electrochemical Detectors

Check that the HPLC pump, detector, and recorder (or integrator) are properly grounded
Use dual-piston reciprocating pumps; a pulse dampener will often reduce background dramatically at high sensitivity ranges
Maintain flow of mobile phase through the detector at all times
Operate at the correct voltage potential; evaluate voltammogram for each cell regularly
Monitor relative peak heights for changes in performance that indicate the need for electrode reconditioning
Store extra reference electrodes in 3 *M* NaCl and replace the reference electrode in the cell 1 to 2 times a week
Disconnect the electrochemical detector when cleaning columns
Use the highest quality water, buffers, and organic solvents
Protect the analytical column and detector with a replaceable guard cartridge

From Binder S. R. and Sivorinovsky G., *Int. Clin. Prod.*, September/October 30, 1986. With permission.

No such simple set of rules can be given to those who wish to develop new methods with EC detection. They should be aware, however, of the possibility of interference from dietary components and pharmaceuticals and their metabolic degradation products. Such interferences may only be occasional and are therefore much less obvious. Binder and Sivorinovsky[45] found, for instance, that their sample prepurification scheme for urinary metanephrines (o-methylated catecholamine metabolites) would not remove synephrine and tyramine, which are found in common foods. Certain adrenergic drugs, e.g., metaproterenol, also coextracted with the metanephrines. All these are phenolic compounds and can be electrooxidized, albeit at slightly higher potential than the metanephrines. The authors suggested the use of test solutions containing these potential interferants together with the metanephrines to aid in setting the optimum working electrode potential.

E. Performance and Role of EC Detection in Neurotransmitter Studies

The great interest of the physiological sciences in neurotransmitters and their metabolites has spurred much activity in analytical methods development. The interpretation of experimental results and clinical studies has been made difficult, however, by a number of factors. Sampling artifacts, low analyte concentrations, small sample weights or volumes, unexpected interferences, a large number of structurally similar analytes,

etc. make it very difficult to judge the adequacy of a method. Furthermore, there are many different analytical problems encountered depending on the sample matrix (brain, urine, serum, etc.), the analytes sought, and the priorities of the user (speed, automation, costs, etc.). All this makes a global judgment of any technique nearly impossible. Nonetheless, LCEC methods are most often quoted as simple, fast, and sensitive. Validation against other known methods has been usually satisfactory, although there remain some differences. Plasma NE values have been found to be much lower by LCEC than by the radioenzymatic method (584 vs. 975 pg/ml).[45a] The difference has been attributed to an unknown plasma constituent, but it could not be established which of the two methods was affected by it.

Opposition to the routine use of EC detection has become stronger when several authors attempted the full automation of catecholamine analysis. Neidhart et al.[45b] have been routinely using the EC method along with radioenzymatic, radiotracer, and chemical reaction detection systems for 6 years. After having analyzed about 80,000 urine samples, they question the utility of EC detection, which they call "a risky adventure", for routine analysis of urine catecholamines. They quote as disadvantages the laborious sample preparation, difficulties of automation, and, particularly, the poorer long-term stability of EC detection systems. Late-eluting irrelevant peaks should be responsible for extended analysis time. These authors propose a combination of column switching technique and postcolumn reaction detection for the automation of urine catecholamine determinations by HPLC. Boos et al.[46] also prefer postcolumn reaction detection for the automated detection of urine and plasma catecholamines. They attribute the following disadvantages to EC detection: unremoved matrix impurities interfere strongly with EC detection; and the column switching and mobile phase changing necessary for automation cause detector stability problems. Neidhart et al.[45b] also suggest that there is no need for the introduction of mass screening for blood catecholamines, where the low detection limits of EC detection might give this technique a leading edge. Urine samples are more easily accessible and are claimed to be of the same diagnostic value in many instances.

The fast development of *in vivo* voltammetry and other *in vivo* analysis methods has raised the question if these methods can replace LCEC in neurotransmitter analysis. Despite the spectacular success of microelectrode measurements in the brain,[47] selectivity and validation problems still must be overcome. Refined techniques of sampling extracellular liquid from the brain now make LCEC measurement viable virtually *in vivo*[48] (Figure 11). LC and direct probing of the brain or body fluids do not appear to be competition for each other at the moment since each has its own virtues: direct probes act fast and on the spot, whereas LC yields more reliable information with respect to speciation. What LCEC users should probably adapt from *in vivo* voltammetry is the successful use of electrode modification and of ancillary electronics to improve or modify

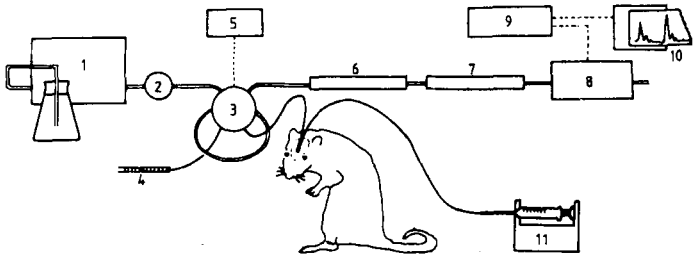


FIGURE 11. LCEC system connected on-line to brain perfusion sampling system. (From Damsma, G., Westerink, B. H. C., de Vries, J. B., Van den Berg, C. J., and Horn, A. S., *J. Neurochem.*, 48, 1523, 1987. With permission).

the selectivity of oxidative detection by carbon electrodes. Further results in fast potential scanning and in microelectrode development might also be useful in LCEC.

LCEC has been used successfully not only to determine the biogenic amines and their metabolites, but also for assays of the enzymes catalyzing their production or degradation in the organism. Figures 6 and 7 have shown the action of these enzymes on the tyrosine and tryptophan metabolites. Some of these enzymes are widely distributed throughout the body. The highest catechol-*O*-methyltransferase (COMT) activity is found in the liver and that of monoamine oxidase (MAO) in the brain and the heart. The activity of these enzymes can be assayed by LCEC since their substrates and/or products are electroactive.

The use of the LCEC technique has only been extended relatively recently to acetylcholine and choline, although acetylcholine is the earliest discovered neurotransmitter and choline is both its precursor and metabolite. The main reason for the delay in the introduction of any HPLC method for these compounds was the lack of a sensitive and selective detection principle. This problem has been overcome by the recent isolation and commercialization of choline oxidase. This enzyme catalyzes the oxidation of choline to betaine:

$$(CH_3)_3N^+CH_2CH_2OH + H_2O + 2O_2 \xrightarrow{\text{choline oxidase}} (CH_3)_3N^+CH_2COOH + 2H_2O_2$$

The produced hydrogen peroxide can be detected amperometrically at a platinum electrode at relatively low oxidizing potential. Acetylcholine can be assayed after hydrolysis to choline by acetylcholine esterase. LCEC systems have been described[48-50] utilizing immobilized acetylcholine esterase and choline oxidase in a postcolumn solid-phase reactor and conventional amperometric detectors equipped with a platinum working electrode. The difficulty in realizing such a system lies in the simultaneous tuning of LC and enzyme reactor conditions to avoid the necessity of a make-up flow between separation and detection. Rat brain and spinal cord dialysates have been analyzed with 50 to 100 fmol detection limits for both analytes. Figure 11 presents one of these systems.

Although EC detectors have been used mainly to detect

electroactive neurotransmitters, there have also been attempts to detect electroinactive neurotransmitters by amperometry after derivatization. In such an attempt, the inhibitory neurotransmitter gamma-amino-butyric acid (GABA) was reacted with 2,4,6-trinitrobenzene sulfonic acid and the derivative chromatographed and detected by reductive amperometry.[51]

IV. PHARMACEUTICALS IN BIOLOGICAL MEDIA

Bulk solution EC methods are quite popular in pharmaceutical analysis. Polarographic methods have been frequently reported [52] along with useful applications of oxidative voltammetry and potentiometry. Flow-through amperometric detectors with some type of carbon working electrode have been successfully applied to pharmaceutical quality control.[5,53] In many pharmaceutical preparations, the active ingredient is the only easily oxidizable compound and chromatographic separation is not required.

Recently, it has become important to monitor the fate of administered drugs. Authorities generally request that the metabolism of new drugs be clarified before they are licensed as pharmaceuticals. With many drugs, successful therapy can only be conducted on the basis of frequent blood or urine controls. To satisfy these needs, pharmacologists have been seeking simple, fast, and sufficiently selective methods. Although GC-MS is generally suitable to solve these problems, it is not accessible in many laboratories and also may require tedious derivatization before analysis. The surprisingly large number of pharmacokinetic studies conducted recently with LCEC seems to substantiate its suitability for this purpose.

Whenever the use of LC has been attempted in pharmacokinetics, the first choice of detector has been the UV detector. However, its sensitivity has been found to be insufficient in many situations. The second choice of most investigators has been fluorimetry or EC detection. Since native fluorescence is relatively rarely observed with the analytes, the choice is between derivatization or EC detection if the latter is feasible.

Musch et al.[54,55] have recently recognized that laboratories spend a lot of time on method development, including the strategic choice between various detectors. They have invoked artificial intelligence to ease this chore. In their quest for an expert system, they have compiled two large sets of experimental data, which may also be useful for those who are not using an expert system but who would like to know if they should attempt LCEC to quantitate a pharmaceutical. The first set of pharmaceuticals was selected on the basis that an oxidative EC response could be definitely expected. This expectation was based on earlier publications proving EC detectability or the presence of phenolic or amine functions. Within this set of 72 drugs, amperometric detection was found to be more sensitive than UV detection for every compound. Interpretation of this statement needs a careful study of the authors' definitions and the method of comparison and relates only to the pure substances and not to biological media. To remain on the safe side, we selected in Table 3 only those substances for which the authors established a gain in sensitivity against UV detection higher than one order of magnitude. The gain was defined as the ratio of minimum detectable concentrations by each method. Hydrodynamic voltammograms of many compounds were also presented by these workers. These curves indicate that some of these substances can be detected at much lower potentials than the tabulated values if the ensuing loss in sensitivity can be traded for the gain in selectivity. The reported minimum detectable amounts by EC were typically about 1 ng injected onto a 250 × 4.0 mm I.D. column. This compares favorably with the 1 to 1000 ng/ml concentrations encountered in pharmacokinetic studies of biological fluids.

The second set of drugs presented by Musch and Massart[55] consisted of 28 drugs selected on the basis of low molar absorbance in the UV range and 15 drugs that are usually present in low concentrations in pharmaceuticals. Both subgroups obviously represent pharmaceuticals for which pharmacokinetic studies by LCUV might be hampered by insufficient sensitivity. The authors used an expert system to predict which of these 43 substances might be amenable to EC detection. The prediction proved to be correct in 40 of the 43 cases. Since the method of prediction was rather simple, those of us without access to artificial intelligence can also use it. One simply has to look in the molecular structure of the drug for the presence of the functionalities shown in Figure 12. In the 94 electroactive drugs of both sets of drugs, only haloperidol, prenylamine, and metamphetamine did not have any of the oxidizable groups of Figure 12. Among the 43 pharmaceuticals with poor UV detectability, clonidine, dihydroergotamine, fenoterol, ethinylestradiol, and lidocaine were detected by EC in two orders of magnitude lower concentrations than by UV.

Drugs that are difficult to detect by either UV or oxidative amperometry were mainly barbiturates, corticosteroids, male hormones, amphetamines, and some antibiotics.

Given the relatively large number of EC-detectable pharmaceuticals with poor UV character, one may expect many more LCEC pharmacokinetic studies to be done. It is useful then to consider what experiences others have had with biological samples. Miner et. al.[56] applied LCEC to the determination of over a dozen pharmaceutically relevant compounds in biological matrices. Successful assay of over 1500 samples convinced these authors of the durability of EC detection. Nevertheless, they also reported difficulties, some of which were not easily surmounted. The relative advantages and disadvantages of EC and UV detection, respectively, as found by these authors are shown in Table 4. The general ways to minimize the electrode coating problem when it occurred were by lowering the amount of compound injected, by decreasing the detector potential, or by including an internal standard with EC properties very similar to those of the analyte. However, these approaches have not always proven to be possible or

Table 3
Pharmaceuticals with Significantly Better Detection Limits by LCEC Than by LCUV

Group	Compound	Gain	Applied potential (V)
Local anesthetics	Benzocaine	36	1.2
	Procaine	25	1.2
	Tetracaine	12	1.2
	Lidocaine	212	1.2
Antipyretics	Acetanilide	18	1.2
	Paracetamol	11	0.9
	Salicylamide	48	1.2
Tricyclic antidepressants	Carbamazepine	20	1.2
	Imipramine	34	1.0
	Desipramine	34	1.0
	Tripramine	25	1.0
	Opipramol	12	1.1
Sulfonamids	Sulfacetamide	18	1.2
	Sulfadiazine	13	1.1
	Sulfaguanidine	27	1.2
	Sulfamerazine	12	1.2
	Sulfathiasol	25	1.2
	Sulfanilamide	32	1.2
	Sulfapyridine	15	1.1
Sex hormones	Dienoestrol	14	1.1
	Estrone	126	1.1
	Ethinyloestradiol	103	1.1
	Hexoestrol	129	1.2
Beta-adrenoceptor blocking agents	Oxprenolol	101	1.2
	Pindolol	36	0.9
	Timolol	19	1.0
	Propanolol	43	1.2
Phenotiazines	Acetophenazine	33	1.2
	Chloropromazine	19	1.2
	Levomepromazine	20	1.2
	Oxomemazine	35	1.2
	Perazine	26	1.2
	Prochloroperazine	28	1.2
	Aminopromazine	23	1.2
	Thioproperazine	28	1.2
	Dimetothiazine	19	1.2
	Thioridazine	22	1.2
Penicillins	Amoxicillin	15	1.2
	Meticillin	35	1.2
	Benzylpenicillin	20	1.2
Diuretics	Triamteren	13	1.2
	Furosemide	23	1.2
	Amiloride	14	1.2
	Hydrochlorothiazide	13	1.2
Alkaloids	Morphine	102	1.2
	Dihydrocodeinone	300	1.2
	Oxycodone	200	1.2
	Dihydromorphinone	140	1.2
	Heroin	34	1.2
	Apomorphine	43	1.2
	Codeine	140	1.2
	Narcotine	100	1.2
	Hydrastinine	23	1.2
	Vindesine	24	1.2
	Vincristine	10	1.2
	Vinblastine	24	1.2
	Desacetylvinblastine	24	1.2

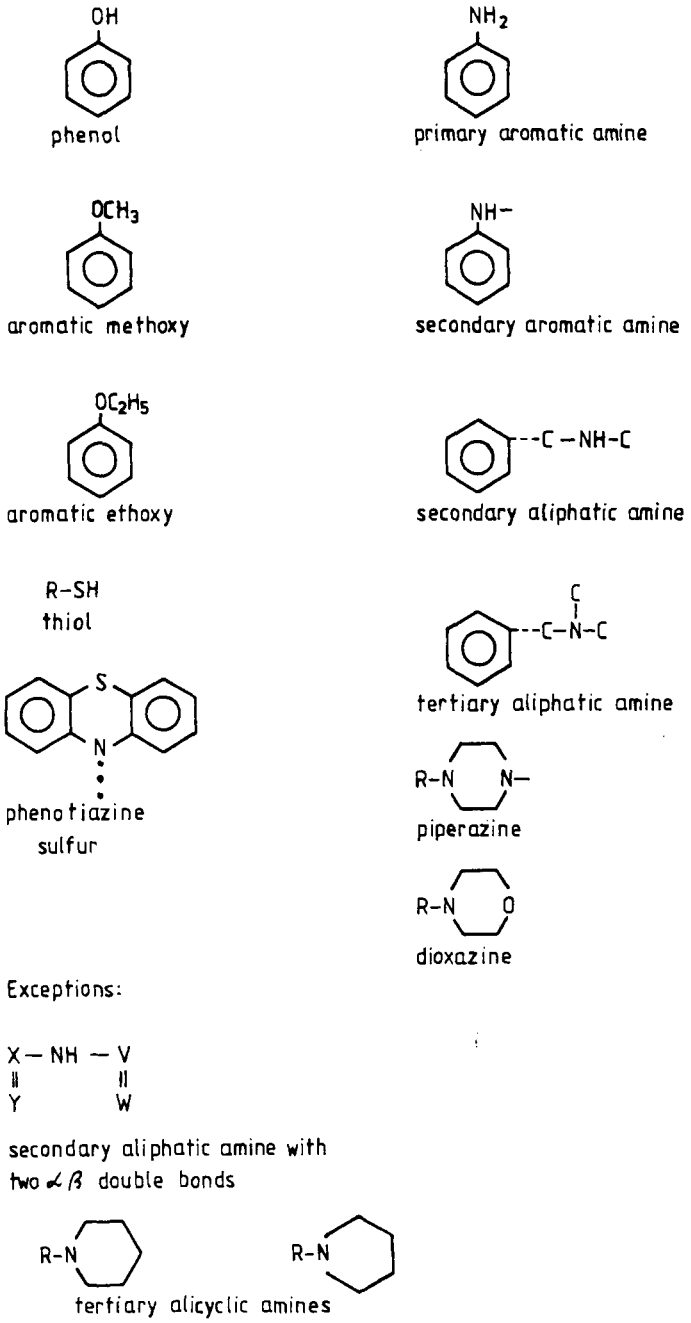


FIGURE 12. Oxidizable functions used to predict the possibility of oxidative amperometric detection. (From Musch, G. and Massart, D. L., *J. Chromatogr.*, 370, 1, 1986. With permission.)

successful. Late eluters were more commonly observed with EC detection than with UV detection. To remove late-eluting, the column-switching technique was found to be rather practical. Using a 3-cm column and an automatic valve, the strongly retained components did not reach the 25-cm analytical column. Not only was sample throughput increased, but surprisingly cleaner chromatograms also resulted (Figure 13). Temperature dependence could be diminished by thermostating

Table 4
Advantages and Disadvantages of EC vs. UV

	EC	UV
Advantages	Sensitivity	Ease of use
	Specificity	Universality
		Isobestic points
		Choice of internal standards
Disadvantages	Coating	Drug interferences
	Reference electrode stability	Temperature sensitivity
	Temperature sensitivity	
	Late eluters	

From Miner D. J., Skibic, M. J., and Bopp, R. J., *J. Liq. Chromatogr.*, 6, 2209, 1983. With permission.

the chromatographic column, working well beyond the half-wave potential, and by using an internal standard with similar diffusional and EC properties to the analyte. At high oxidation potentials, baseline instabilities were observed which could be eliminated by switching to another source of methanol in the eluent. Addition of EDTA to the mobile phase or using phosphate/citrate buffers was recommended to suppress baseline oscillations due to heavy metals dissolved from the chromatographic system.

Miner et al.[56] also attempted to establish a guideline for decision between UV and EC detection. They suggested that the EC detection limit for the pure substance, i.e., matrix effects, filming, etc. neglected, will be lower than the UV detection limit if the molar absorptivity is low and the half-wave potential is not too high. Their graphic dividing line was approximately

$$A = 45000 - 35000\ E_{1/2}$$

where A is the molar absorptivity in liters per mole per centimeter and $E_{1/2}$ is the half-wave potential in volts.

A survey of a number of publications on LCEC detection of pharmaceuticals in biological media reveals that sample pretreatment and the choice of the mobile phase are extremely important to obtain sufficiently clean chromatograms. It has often been noted that the detection limits are much higher when biological media rather than pure substances are analyzed. Much effort and time could apparently be saved if suitable guidelines were developed for pharmacologists to assist them in cleaning their biological samples for LCEC. The great potentials of good sample pretreatment can be judged from the excellent results obtained in the determination of biogenic amines from body fluids. One key to success here was the vast amount of research that went into sample prepurification.

When looking for a general pattern in the publications on pharmaceuticals determination by LCEC in biological media, a number of important steps could be discerned in method development. Obtaining a hydrodynamic current-voltage curve of the pure substance is indispensable. The effect of pH on the

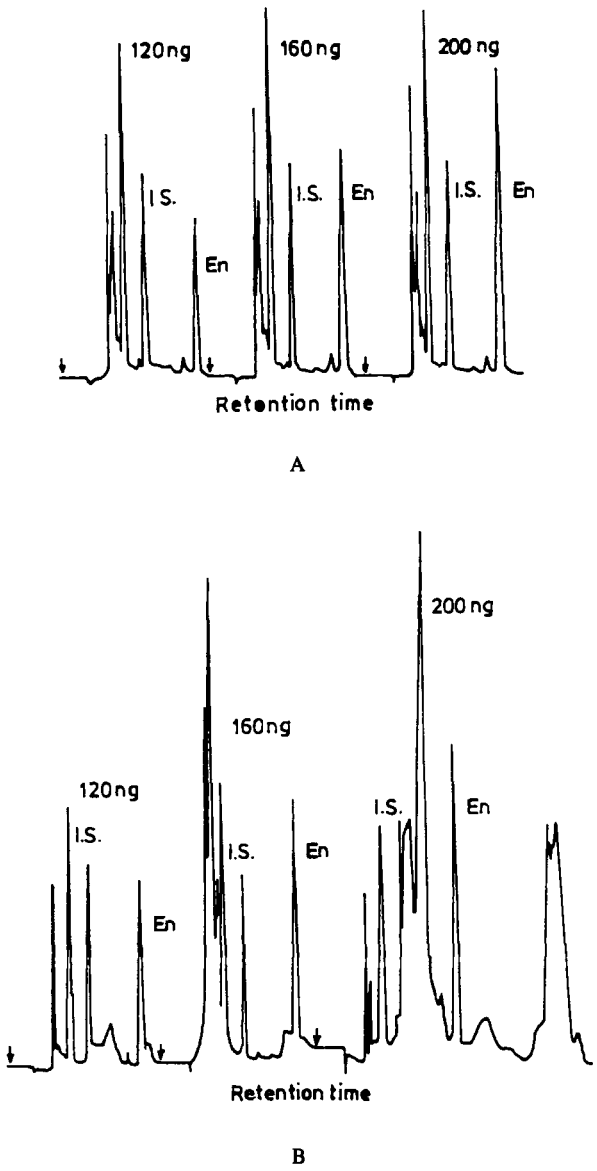


FIGURE 13. Chromatograms of plasma extracts spiked with the drug enviradene (En) and internal standard (I.S). (A) Plasma extracts with column switching; (B) plasma extracts without column switching. (From Miner, D. J., Skibic, M. J., and Bopp, R. J., *J. Liq. Chromatogr.*, 6, 2209, 1983. With permission.)

electrochemistry and retention is usually rather marked. Analyte extraction and eventual preconcentration is still most often done by liquid-liquid extraction, although solid-phase extraction might be more suitable. Decomposition of the analyte during sample storage and preparation has been frequently observed. A sufficiently representative number of blank samples should be analyzed from humans or experimental animals not treated by the drug to be studied. In this way, endogenous interferences can be sorted out. Possible metabolites should be checked for interference with the analyte. Regarding humans, interference from other drugs that they are likely to use and from the metabolites of these drugs needs to be excluded. The choice of internal standard is of the utmost importance. It should resemble the analyte with respect to both the separation processes (sample purification) and EC behavior. The method developed should be carefully characterized in terms of linear range, detection limit, interassay variability, intra- and interday assay precison, etc. Repetitive injections often reveal slow electrode fouling, but frequent standardization may help somewhat in this respect.

LCEC detection of pharmaceuticals in biological media has not been limited to the conventional single working electrode oxidative voltammetry. Concurrently, the whole instrumentation of EC detection has been used. A fast dropping mercury electrode and a gold amalgam electrode cell were compared for instance by Kok et al.[57] Tjaden et al.[58] evaluated a mercury electrode cell in the hanging drop and the static mercury drop working mode. Hageman et al.[59] used a rotating working electrode in a wall-jet cell. Spreux-Varoquaux et al.[60] utilized a dual coulometric cell; Isaksson and Kissinger[61] used a series dual-electrode amperometric cell. Iodine or bromine generated electrochemically from the iodide or bromide containing eluent in a postcolumn reactor has also been used to oxidize the analytes.[57,62] The excess halogen was subsequently detected by amperometry.

V. FORENSIC ANALYSIS BY LCEC

There are two areas of forensic analysis where LCEC has been extensively tested and used: explosives and drug abuse.[63,64]

Explosives and firearms propellants are of similar composition, typically containing compounds belonging to the nitramines, alkyl nitrates, or aromatic nitro compounds. All of these are readily reduced at mercury or gold amalgam electrodes, and they are also easily separated by LC. Samples are typically traces on handswabs that may indicate whether or not people and their possessions have been in contact with explosives. At the scenes of bombings, the identification of the type and manufacture of the explosive is attempted after vapor-phase sampling. The latter are very clean samples, whereas traces collected on swabs from skin need extensive clean-up by solid-phase extraction.[65]

The residues from firearms propellants are remarkably stable.[63] Experiments have been conducted in which a single shot was discharged from a revolver and 7 h later nitroglycerin could be detected even on washed skin surfaces. At that time, 0.2 to 1.4 ng were collected per swab. Clothing is a still better source for sampling: 80 ng of nitroglycerin was retrieved from an outer garment after 5-d wear following the exposure at a revolver shot.

After clean-up, samples are subjected to reversed-phase chromatography. Since reductive detection is used, the eluent needs to be deoxygenated, e.g., by gentle reflux and the samples are deoxygenated by using a slight modification of the injector.[66] PTFE tubing needs to be replaced by stainless steel

to avoid oxygen reabsorption from the ambient air. Cathodic potentials of 0.9 to 1.0 V vs. a Ag/AgCl reference electrode are used. Residual currents are a factor of 2 to 20 times the analyte peak height at the nanogram level. If a new oxygenated column is installed, then several hours of purging is required before a stable baseline is obtained.

Nitrocellulose is often used in firearms propellants. It is reduced on mercury near 0 V vs. the Ag/AgCl electrode. When size exclusion chromatography is combined with reductive EC detection, propellants can be readily differentiated.[67] Stabilizers in propellants like diphenylamine may be detected along with the propellants in a dual detector system utilizing an oxidative coulometric detector upstream and a mercury drop electrode downstream.[68] Determination of gunpowder stabilizers by LCEC and concurrent atomic absorption analysis for trace metals yielded substantial evidence as to the presence of gunshot residues.[69]

Outside of the forensic laboratory, munitions components also need to be detected in the environment, mainly in surface and groundwater. The source of contamination is the munitions industry. Maskarinec et al.[70] reported the development of a resin-based isolation scheme for a wide range of munitions components and byproducts from water samples. This pretreatment was combined with reductive LCEC using a gold amalgam thin-layer electrode and following the recommendations of Bratin et al.[71,72] Detection limits approaching 1 ng/ml were obtained for many compounds.

Reductive EC is very suitable for detection of explosives and oxidative EC directly does not apply. Explosives can be transformed, however, by postcolumn UV irradiation to oxidizable products and these can be detected by oxidative EC.[73]

Drug abuse is frequently analyzed by forensic laboratories. Samples include bulk pharmaceuticals, illicit preparations, and biological fluids. Thin layer and gas chromatographic methods are commonly utilized and are generally adequate. For thermally unstable or relatively nonvolatile compounds, LC might offer some advantages. It could also serve to confirm other results. Reports of LCEC to detect drug abuse have been reviewed by Selavka and Krull.[64] These authors found in the literature up to 1986 about two dozen articles related to the subject. Half of these discussed morphine and apomorphine determinations, and many of the remaining articles dealt with determinations of benzodiazepines and other drugs in biological samples in general, not necessarily for forensic purposes. Selavka and Krull concluded, probably correctly, that the potential of using LCEC to identify and quantitate controlled substances is much bigger than its present use. Morphine and several other narcotics can be detected with excellent sensitivity and selectivity by oxidative LCEC in biological fluids and tissues. Cannobinoids[74,75]and tryptamine-derived hallucinogens may also be oxidized electrochemically, although this latter group has not been sufficiently tested yet. Benzodiazepines are well suitable for reductive detection. Postcolumn UV degradation followed by oxidative dual-electrode detection has been put to the test with different problems. It appears to offer sufficient selectivity for screening illicit cocaine samples and sufficient selectivity and sensitivity to become the LC detector of choice for barbiturates and benzodiazepines.

VI. DERIVATIZATION AND POSTCOLUMN REACTIONS

Reductive and oxidative EC detectors offer selective detection with rather low detection limits. Their selectivity toward electroreducible or oxidizable analytes is one of their main strengths, but it is of course also a serious limitation. An elegant way to extend the high sensitivity and selectivity to electroinactive analytes lies in transforming them by selective chemical reactions into equivalent amounts of an electroactive substance. This can be done by pre- or postcolumn derivatization with electrophores or by performing a postcolumn reaction between the analyte and a reagent leading either to a measurable loss of electroactive reagent or producing an electroactive byproduct. These possibilities are explained by examples discussed in the following section.

One strength of derivatization is in the selectivity of the chemical reaction. A further increase in selectivity can be expected by using electrophores reduced or oxidized at rather low potential. This could eliminate some matrix interferences. The drawbacks of derivatization are also obvious. Precolumn derivatization, usually made off-line, requires extra manual work. Postcolumn derivatization systems are more convenient but also more expensive. Development of new derivatization methods, suitable for EC detection, is certainly tedious work and requires preparative skills. Derivatization reactions should be fast, use easily accessible and stable reagents, and lead to unique, stable products. When precolumn derivatization is made, the products of different derivatized molecules should remain sufficiently different to make chromatographic separation with a reasonable plate number possible.

A unique method to convert electroinactive analytes into electroactive ones after separation has been used by Krull and co-workers[73,76-78] to determine many groups of analytes. They interposed a UV irradiation flow-through reactor between the analytical column and the oxidative EC detector. Photolysis frequently produced electrooxidizable molecules from the analytes, e.g., organic nitro compounds, organothiophosphate pesticides, penicillins, barbiturates, cocaine, benzodiazepines, organoiodides, etc. Figure 14 shows the response of a parallel dual electrode detector to a sample serum containing butabarbital with the UV lamp on in one case and off in the other. Both butabarbital and the internal standard were only detected when the lamp was on. Righezza et al.[79] applied the method to nitrosamines analysis. Krull and co-workers also succeeded in applying this method to a variety of sample matrices: malathion was determined in wheat samples, bacampicillin hydrochlorid in formulations, chlordiazepoxide in human urine,

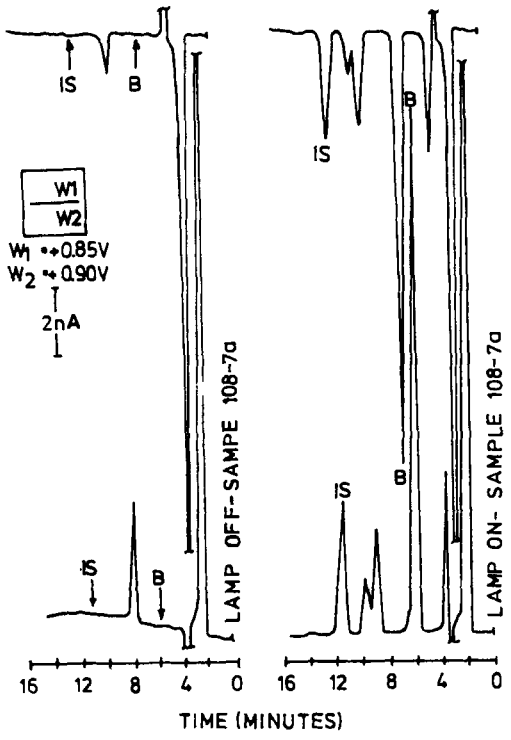


FIGURE 14. Determination of butabarbital (B) and internal standard (IS) in serum with postcolumn photochemical reaction (see text). (From Krull, I. S., Selavka, C. M., Duda, C., and Jacobs, W., *J. Liq. Chromatogr.*, 8, 2845, 1985. With permission.)

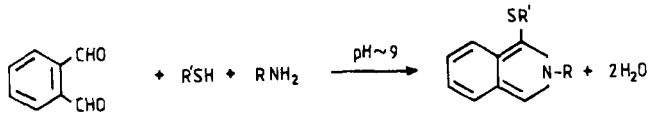
barbiturates in human serum, etc. These validation studies are quite important since many matrix components might in principle also be degraded by UV light and produce interfering peaks.

The encouraging results obtained by many in derivatization for LCEC have been carefully reviewed recently[77] and therefore only a brief overview is given here. General methods of derivatization for LC can be found in References 80 and 81.

A. Precolumn Derivatizations

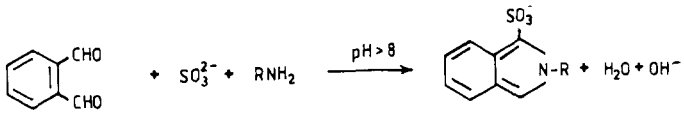
Many of the derivatization reagents used for UV or fluorescence detection also yield electroactive products. Nitroaromatics are very suitable for reductive detection, whereas o-phthalaldehyde (OPA) derivatives of amines can be electrooxidized. Nambara and co-workers developed a new family of electroactive derivatizing reagents based on ferrocene. These reagents were used to label amines,[82] hydroxy compounds,[83] and thiols.[84]

Amines have been derivatized by a variety of reagents. Jacobs and Kissinger[85] used different nitroaromatics and found trinitrobenzene sulfonic acid to perform best. Caudill et al.[51,86] used the same reagent and could determine GABA in rat brain homogenates. OPA has been used by several authors to determine amines and amino acids by oxidative amperometry. The derivatization reaction requires a co-reagent. This is usually a thiol:



Scheme I

Allison et al.[87] found that derivative stability was satisfactory when t-butyl thiol was used. More recently, Jacobs[87a] suggested the use of sulfite instead of a thiol to further improve product stability and to avoid the undesirable odor of thiols:



Scheme II

There have been also several reports on derivatization of isocyanates[88,89] and cyanide.[90] Metal ions may be complexed to reagents dissolved in the mobile phase or in a precolumn reaction and the complex oxidized in the detector. Bond and Wallace[91,92] determined copper, nickel, cobalt, chromium (III), and chromium (VI) using dithiocarbamate complexes. Aldehydes and ketones may be determined by reductive LCEC as dinitrophenylhydrazones,[93] but the phenylhydrazones are also amenable for oxidative detection.[94,95]

B. Postcolumn Reactions

Postcolumn derivatization by chemical reactions appears to be used infrequently in LCEC. Little et al.[96] reported the determination of oxo compounds by admixing the semicarbazide reagent postcolumn. Honda et al.[97] determined glucose and other reducing carbohydrates by postcolumn derivatization with 2-cyanoacetamide.

Much more interest has been shown in nonderivatization-type postcolumn reactions. These might also be called indirect detection methods because the loss of reagent or the concentration of a byproduct of the reaction is measured. Similar methods had been used in flow injection analysis before they were applied to LC detection.[5] King and Kissinger constructed a postcolumn device where *in situ* electrogenerated bromine reacted with the separated analytes and the decrease in bromine level was measured amperometrically.[98] They determined nanogram levels of fatty acids, prostaglandins, and phenols. Kok et al.[99] brominated phenolic ethers in a similar way and obtained improved selectivity in the determination of *N*-acetylcysteine by using electrogenerated iodine as the postcolumn reagent.[57] In these systems, the precursor of the reagent (bromide or iodide) is dissolved in the mobile phase

and a flow-through coulometric-type generator cell is connected postcolumn to produce the reagent. The reagent can also be admixed to the eluate. Watanabe and Inoue[100] added copper phenanthroline reagent to reducing sugars eluting from the separation column. The reduced reagent was detected in the oxidative amperometric detector cell.

Enzymatic postcolumn reactions are similar in principle to the chemical reactions discussed earlier. For example, acetylcholine and choline have been determined by homogeneous[101] or heterogeneous[49] postcolumn enzymatic reactions. Oxygen, dissolved in the eluent, was reduced to hydrogen peroxide in the enzyme reaction and hydrogen peroxide was detected by oxidation at a platinum electrode. Postcolumn enzymatic reactions combined with EC detection have also been used to determine bile acids,[102] phenolic glycosides,[103] and cyanogenic glycosides.[104] Taylor and Nieman[105] used bipolar pulse conductivity measurement to detect changes in ionic concentration resulting from postcolumn enzymatic oxidation of amino acids.

VII. ELECTROCHEMICAL DETECTION IN MICROCOLUMN AND HIGH-SPEED LC

In recent years, there has been an important trend in LC to use either narrower or shorter columns than the 25 cm × 4.6 mm I.D. columns used most frequently. Both types are claimed to produce minimal dilution of the injected sample, therefore the common term low-dispersion LC also applies.

Short columns are typically 5 cm in length. Their I.D. is conventional, i.e., 4.6 mm. They are packed with 3-μm spherical particles and the flow rates used are 2.0 to 5.0 ml min^{-1}. Reduction of the particle size to 3 μm provides two main advantages: the column length required to achieve a given efficiency decreases and the optimum mobile phase velocity increases. In other words, shorter columns at higher flow rates achieve similar separations as conventional (25 cm × 4.6 mm, 5 to 10 μm) columns. The obvious result is that analysis time is considerably reduced. To keep pace with the improvement in separation bandwidth, detectors with lower dispersion are also required.

The trend to decrease the LC column diameter has led to three different types of separation systems: small-bore, packed-capillary, and open-tubular capillary columns. Figure 15 and the data in Table 5 clearly indicate what these names involve. Microcolumn methods allow very high plate numbers to be achieved at usual working pressures. Furthermore, typical flow rates are greatly reduced compared with conventional LC, and this results in solvent savings and in the possibility of using exotic mobile phases. Microcolumn methods like fast LC reduce band spreading on the column. Extracolumn band broadening has to be reduced correspondingly. This means that smaller volumes need to be injected and the detector volume has to be reduced rather significantly (Table 5).

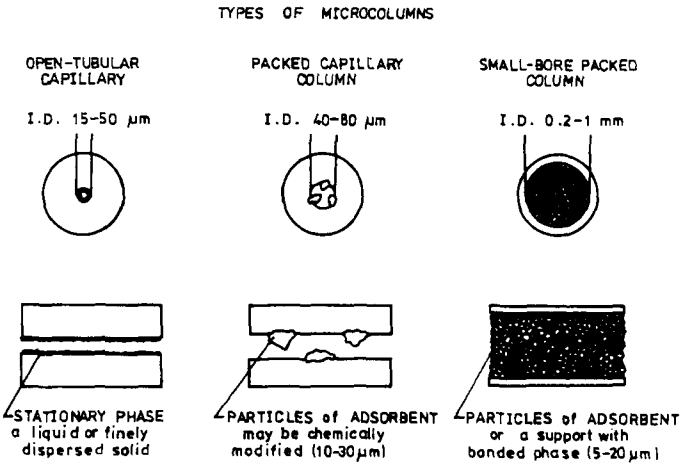


FIGURE 15. Types of LC microcolumns. (From Novotny, M., *Microcolumn Separations*, Vol. 30, Novony, M. and Ishii D., Eds., Elsevier, Amsterdam, 1985, 19. With permission.)

It can be seen that both trends lowering dispersion in LC have led to the need for detectors with orders of magnitude lower detector volumes than conventional flow-through UV cuvettes (~ 10 μl). It has been recognized by many that EC detectors are much more easily miniaturized without a loss in concentration sensitivity than spectroscopic cells, where smaller volume usually means shorter light path and decreased sensitivity.

Some interesting examples of using EC detectors in low-dispersion LC are shown in the following section. It is noted, however, that in these microcolumn applications the separations and detection limits realized are not spectacularly better than in conventional size systems, and sometimes they are even worse. It remains to be seen what progress the future brings in this developing area.

Some of the commercial EC detectors are suitable for fast LC or micro-LC work with, at most, minor modifications. This has been shown for example with the thin-layer cells of Bioanalytical Systems (West Lafayette, IN). Di Bussolo et al.[107] replaced the pastic top half cell of the cell block with stainless steel and used it as the auxiliary electrode (this modification appears to be the standard by now). They could substantially decrease the bandwidth in this way (from 164 to 64 μl). It has been calculated that the loss in plate number would only be about 10% on a 15,000 theoretical plate column for a not too early eluting peak (k' = 3). The authors pointed out that further improvement was possible but the detector was already suitable for use with 100 × 4.6 mm columns packed with 3-μm particles.

Caliguri et al.[108] tested a similar thin-layer cell with a stainless steel top half and a very thin, 10-μm spacer. The geometric cell volume was 0.2 μl. These authors used high-speed small-bore columns, i.e., short (10 cm) and small bore (1.2-mm I.D., 8 μm reversed-phase packing) columns. The exit end of

Table 5
Typical Data for LC Columns

	Column I.D. (μm)	Particle size (μm)	Column length (m)	Flow rate (μl/min)	Injection volume* (m)	Peak volume (μl)	Detector volume* (μl)
Conventional	4600	5	0.25	1000	10	500	10
Small bore	200—1000	5	1	10	1	5	1
Packed capillary	40—80	30	10	2	0.1	1	0.1
Open tubular capillary	15—50	—	10	0.1	0.001	0.01	0.001

* Typical maximum injection or detector volume calculated for optimum velocity and plate height and allowing about a 5% increase in column variance in each case.

the column was directly seated in the entrance port of the stainless steel block of the amperometric cell into which a stainless steel frit had been placed. Typical plate counts of the system were about 5000 at the normal operating flow rate of about 200 μl min^{-1}. A high-speed small bore separation is shown in Fugure 16. The authors claimed 1 pg or lower detection limits for biogenic amines. The speed of separation and the possibility of injecting up to 5-μl sample with virtually no peak distortion at $k' > 1$ are notable in this system. The same type and size of column were also used by Wages et al.[109] in conjunction with a similar thin-layer cell. When the channel in the spacer was 3 mm wide and 20 μm thick, the cell volume became 0.96 μl. This cell volume gave a signal 2.5 times larger than a cell of 1.4 μl. The detection limit for dopamine was 300 fg, about 16-fold better than with a 4.6-mm column of the same (10 cm) length and packed also with 3-μm particles.

Many miniaturized amperometric cells have been constructed. These have been recently reviewed by Slais[110] together with other EC detector types used for small-bore work. One of the interesting developments has been the development of single and dual working electrode thin-layer cells by Goto.[111] The typical advantages of these cell types were realized with 0.06- to 1.0-μl cell volumes. Moreover, with parallel-opposed dual electrodes of 10 × 2 mm^2 area (Figure 17), current amplifications of up to 20-fold were achieved. The current amplification factor is the ratio of the observed current to the current expected for 100% conversion. Amplification was due[112] to repetitive oxidation-reduction cycling of the analyte molecules during passage through the cell. Very low flow rates (1.4 to 11.2 μl min^{-1}) were used with the 0.5-m I.D. columns and this led to lengthy separations in some cases.

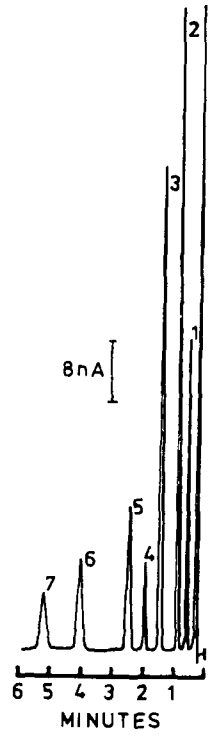


FIGURE 16. High-speed, small-bore resolution of biogenic amines. Peak identities: 1 — DOPEG, 1.1 ng; 2 — NE, 2.06 ng; 3 — DOPA, 3.31 ng; 4 — E, 0.79 ng; 5 — DHBA and 6 — DOPAC, 1.34 ng; 7 — DA, 0.97 ng. (From Caliguri, E. J., Capella, P., Bottari, L., and Mefford, I. N., *Anal. Chem.*, 57, 2423, 1985. With permission.)

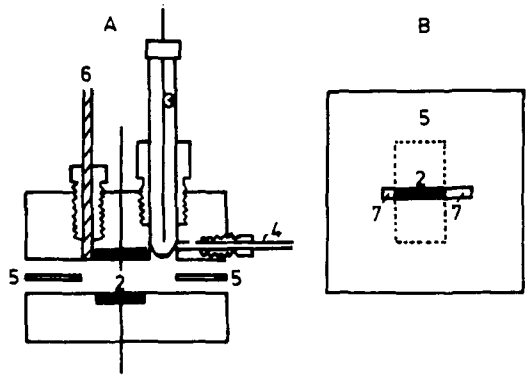


FIGURE 17. Cell for voltammetric detector with two working electrodes in parallel-opposed configuration. (A) Side view of cell; (B) front view of spacer. (1 and 2) Working electrodes (glassy carbon); (3) reference electrode (Ag, AgCl); (4) counter electrode doubling as outlet (Pt tube); (5) spacer (Teflon sheet, 30 to 50 μm); (6) microseparation column; (7) slot. (From Goto, M., *Microcolumn Separations*, Vol. 30, Novotny, M. and Ishii, D., Eds., Elsevier, Amsterdam, 1985, 309. With permission.)

Whereas conventional amperometric cells with minor modifications could be successfully used in fast LC and small-bore LC, open tubular capillary columns posed a more delicate situation. Due to the recent development of using micron-sized electrodes, this problem could also be solved. Knecht et al.[113] constructed a detector by carefully inserting the tip of a 9-μm diameter carbon fiber working electrode into the end of the column (15-μm I.D.) with a micropositioner. One of the advantages of using a microelectrode has been that fairly fast potential scanning could be used[114] and three-dimensional chromato-voltammograms resulted.

Conductivity cells for small bore ion chromatography have been constructed. Here again the analytical column or the suppressor is at best directly connected to the inlet port of the cell. Rokushika et al.[115] used the simplest conceivable cell consisting of two short lengths of stainless steel tube connected in series into the flow line and separated by a PTFE sleeve at a distance of 0.5 mm. Slais[116] used a dual-purpose conductometric-amperometric cell of 15-nl volume.

Potentiometric sensors are not frequently used for detection in LC. They are, however, well suited for miniaturization, and ion-selective electrodes with micron-sized tips are routinely used in the biological sciences. Simon and co-workers[117,118] demonstrated the use of micro-ionselective electrodes for on-column detection in open tubular column LC. They have shown that the electrode need not even be inserted into the column end but it can work as a wall-jet electrode when positioned with its tip in front of the capillary column's exit orifice.

VIII. ELECTROCHEMICAL DETECTION IN ION CHROMATOGRAPHY

The term ion chromatography was introduced only in the last decade when Dionex Corp. began to market a proprietary chromatographic system based on the work of Small et al.[119] Although the separation process applied was not different from classical ion exchange, very important innovations were made. To make universal detection by conductivity measurement possible, the high background conductivity caused by the then typical eluents had to be eliminated. This goal was achieved by developing new, low capacity anion exchangers for the separation and by neutralizing the $NaOH/Na_2CO_3$ eluent in a postcolumn reactor, called a suppressor, to water and carbonic acid, respectively. Low capacity ion exchangers could be operated with low eluent concentrations. Water and carbonic acid contribute very little to eluate conductivity, whereas the sample anions emerge from the suppressor as the respective acids, i.e., in a highly conductive form unless the acid was rather weak. Later, Gjerde et al.[120] introduced another detection technique also based on ion separation on low capacity ion exchangers. In their "single-column" system, organic eluent ions with low equivalent conductivity but high affinity for the ion exchanger were used. Combined with a rather sensitive conductivity detector, this system allows comparable performance to the suppressed one. Today, the term ion chromatography is more widely understood — it refers to any efficient method of separating and determining ions,[121] including ion pair reversed-phase chromatography. It appears to include any separations on ion exchangers, e.g., ion exclusion chromatography is also covered in recently published books and reviews.[121-125] Recent developments in detection methods for ion chromatography have been reviewed by Haddad.[126]

Three types of electrochemical detectors have been used in ion chromatography: conductivity, voltammetric, and potentiometric detectors. Before turning our attention to these individual types of detectors, it is useful to consider what is actually the measured quantity in ion chromatography. For the sake of simplicity, only anion exchange is considered and it is assumed that all considered species are fully ionized. The chromatographic process in anion chromatography is based on the competition between analyte anions A^- and eluent anions E^- for the covalently bound cationic sites R^+ on the resin:

$$R^+E^- + A^- = R^+A^- + E^-$$

This exchange reaction keeps the total concentration of anions in the mobile phase constant, only the ratios of E^- to A^- will change along the column.

The capability of the eluent to drive the analyte ions down the column is due to either its higher affinity for the binding sites and/or its higher concentration relative to the analyte.

The detector is, in general, sensitive to both A^- and E^-, but the sensitivity factors (detector signal change per unit equivalent concentration) are usually different. In fact, if they were not different there would be no peaks on the chromatogram since the total concentration does not change. The concentration vs. time profiles of A^- and E^- when the peak is passing at the detector are shown in Figure 18. If detector sensitivity for A^- is s_{A^-} and for E^- is s_{E^-}, then the signal (S) is

$$S = C_{E^-}s_{E^-} + C_{A^-}s_{A^-}$$

The background signal is

$$S_B = C_{tot}s_{E^-}$$

where C_{tot} is the total (anion) concentration. The signal change from the background is

$$S - S_B = (C_{E^-} - C_{tot})s_{E^-} + C_{A^-}s_{A^-} = C_A(s_{A^-} - s_{E^-})$$

because

$$C_{tot} = C_{E^-} + C_{A^-}$$

This calculation shows that the peaks may be positive or negative deflections from the baseline depending on the relative sensitivities. If $s_{A^-} > s_{E^-}$, the peaks are positive deflections,

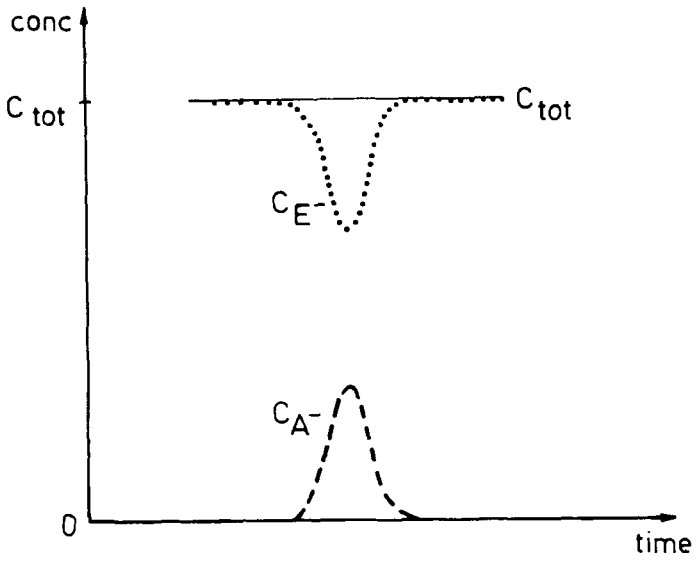


FIGURE 18. Concentration-time profiles of analyte (A^-) and eluting (E^-) ion in the mobile phase as observed by the detector.

otherwise they are negative, and there is no peak for $s_{A^-} = s_{E^-}$.

It has become customary to speak of direct detection when all peaks are signal increases. This implies that detector sensitivity for practically all analytes is higher than for the eluent. For instance, this has been realized in conductivity detection and single-column ion chromatography. When conductivity is measured, the sensitivity to each ion is proportional to its equivalent conductance. In single-column ion chromatography, some of the useful eluents are salts of bulky organic acid anions with rather low equivalent conductance (s_{E^-}). Practically all analyte anions have higher equivalent conductance (s_{A^-}).

The detection mode has been called indirect when all peaks are negative deflections from the baseline, i.e., the detector is more sensitive toward the eluent than toward any of the analytes. Taking single-column anion chromatography with conductivity detection as an example again, the eluent may be a strong base, like NaOH. The equivalent conductance of the hydroxide ion is well known to exceed that of other anions.

Apparently, any detector can be used for both direct and indirect detection if suitable chromatographic conditions can be found. The requirements toward detector performance are usually more stringent, however, with indirect detection. In this mode, the baseline is, per definition, at a relatively high signal level. With most detectors (potentiometric sensors in certain concentration ranges are exceptions), higher signals are noisier. This can make low detection limits more difficult to achieve. However, the higher noise level may be compensated for by higher sensitivity. Comparing the situation in the two earlier examples, one finds that with the hydroxide eluent the sensitivity for typical analytes like the halides is three to seven times higher than with the organic anion eluents.

A. Conductivity Detection

Since this general discussion dealt with conductometric examples, only some special subjects need to be considered. here.

Instrumentation for conductivity measurement in ion chromatography is rarely discussed, although there are many different cell constructions commercially available. These appear to have been designed on the basis of known principles. Low and high frequency methods have been used as well as direct current measurement with a four-electrode cell. Dionex uses the bipolar pulse method.[127] The detectors offered for single-column ion chromatography are usually well thermostated and offer the possibility of measuring conductivity changes of 1 in about 10,000. Very recently, Jen et al.[129] suggested the use of derivative conductometric detection for better peak discrimination. Conductivity instrumentation has been reviewed in more detail.[128]

Gradient elution has generally not been used in ion chromatography, perhaps because isocratic separations were satisfactory in many situations. If ions with widely differing retention (very different valencies) are present in the sample, gradient elution may be necessary. Rocklin et al.[130] found that, among different possibilities for gradually increasing the eluent strength, concentration gradients are best suitable when conductivity detection is used in a suppressed system. Eluent impurities may be rather disturbing, and these authors found an elegant way to circumvent the problem. Figure 19 shows separation and elution of 36 ions in 30 min by the gradient method.

Separation of weak acids on ion exclusion columns and detection by conductivity is an interesting application of ion chromatography. Recently, McCrory-Joy[131] used a resin-based anion column with a basic eluent and conductivity detection without suppression to separate weak acid anions.

Determination of nonionic substances by conductivity detection has been a challenge for many. Tanaka and Fritz[132] used ion exclusion chromatography on a cation-exchange column (hydrogen form) with a dilute aqueous solution of sulfuric acid as the eluent to separate alcohols, sugars, and carboxylic acids. The latter gave positive peaks at the conductivity detector while the nonionics gave negative peaks. Stevens et al.[133] determined water using LC with conductivity detection. The Karl Fischer method and gas chromatography are sometimes not usable because of interferences and this has justified development of the new method. The eluent was an organic solvent, mostly methanol with about 0.001 *M* strong acid dissolved in it. A short ion exchange column was used and water eluted within 5 min, usually as the latest peak. The detection limit was 2.5 ppm water. In another approach for nonionics,[134] carbohydrates were determined by ion exchange chromatography using a boric acid solution as eluent. The carbohydrate-boric acid complexes are considerably stronger acids than boric acid and conductivity detection is possible.

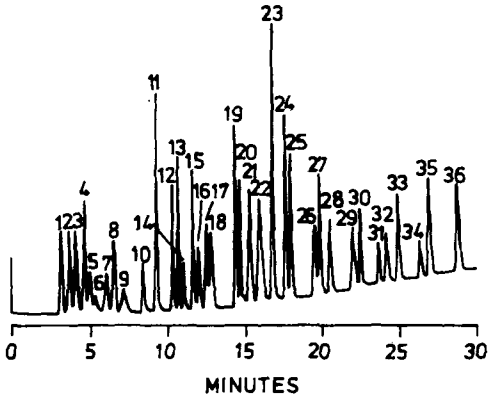


FIGURE 19. Gradient elution of inorganic and organic anions. All anions are 10 ppm unless otherwise noted. Peak identification: (1) fluoride (1.5 ppm); (2) α -hydroxybutyrate; (3) acetate; (4) glycolate; (5) butyrate; (6) gluconate; (7) α -hydroxyvalerate; (8) formate (5 ppm); (9) valerate; (10) pyruvate; (11) monochloroacetate; (12) bromate; (13) chloride (3 ppm); (14) galacturonate; (15) nitrite (5 ppm); (16) glucuronate; (17) dichloroacetate; (18) trifluoroacetate; (19) phosphite; (20) selenite; (21) bromide; (22) nitrate; (23) sulfate; (24) oxalate; (25) selenate; (26) α -ketoglutarate; (27) fumarate; (28) phthalate; (29) oxalacetate; (30) phosphate; (31) arsenate; (32) chromate; (33) citrate; (34) isocitrate; (35) cis-aconitate; and (36) trans-aconitate. The following eluents were used: eluent A, 0.75 m*M* sodium hydroxide; eluent B, 100 m*M* sodium hydroxide. Gradient program:

Time (min)	0	5	15	30
%A	100	100	70	14
%B	0	0	30	86

Flow-rate, 1.0 ml/min; 30 μS full scale range; sample loop, 10 μl. (From Rocklin, R. D., Pohl, C. A., and Schibler, J. A., *J. Chromatogr.*, 411, 107, 1987. With permission.)

B. Voltammetric Detection

The early separations of biogenic amines and also some recent ones have been carried out on ion exchange columns. Therefore, voltammetric detection after ion exchange separation has a long tradition. Within the realm of ion chromatography, the most important application has been the detection of weakly dissociating species in suppressed methods. Suppression transforms the separated anions into acids and these are not detected by the conductivity detector if they are not dissociated, like the acid forms of cyanide, sulfide, and arsenite. Interposing an oxidative voltammetric detector between the analytical column and the suppressor, these ions and some others can be sensitively detected. In contrast to the oxidative determination of organics where carbon electrodes are preferred, here the electrode material has been usually silver or platinum. With the silver anode, the analyte is normally not oxidized itself. Rather the silver anode is oxidized to form an insoluble sulfide or soluble cyanide complex. The rate of oxidation, i.e., the current, is determined however by the rate of diffusion of the analyte to the electrode surface. Therefore, the current is proportional to analyte concentration. Some of the other ions like bromide and iodide are detected both by this detector and the conductivity detector.

A thorough investigation of anodic detection in ion chromatography at the silver electrode has been published by Rocklin and Johnson.[135] They reported detection limits of 2, 30, 10, and 10 ppb for cyanide, sulfide, iodide, and bromide, respectively. These values have been claimed to be orders of magnitude lower than what could be achieved when the analytes were oxidized themselves on a gold electrode or when a mercury anode was used.[136] The method can be used to determine free cyanide. Total cyanide can also be measured after acid decomposition of the sample and distillation of cyanide. Han and Koch[137] were able to lower the detection limit for sulfide to 0.1 ppb by eliminating difficulties due to eluent contamination and to trapping of some sulfide on the column. Han et al.[138] also optimized the determination of iodide by ion chromatography with amperometric detection. With suitably pretreated platinum electrode to oxidize iodide, they obtained better linearity at low concentrations (10 to 100 ppb) than the earlier investigators[135] using silver working electrode. An ion exclusion chromatographic method for sulfite in foods with oxidative detection at a platinum electrode has been claimed[139] to be faster, more sensitive, and more interference-free than other current methods. Features and applications for a commercial amperometric detector in ion chromatography have been discussed recently.[140]

Amperometric detection has sometimes been used in combination with conductivity detection. Slais[116] constructed a 15-nl microcell where the same pair of electrodes is used for DC amperometry and AC conductivity measurement.

Carbon electrode materials are not particularly useful for direct determination of chromatographically separated simple inorganic ions. It has been shown, however, that indirect detection is possible if the eluent is electroactive at the carbon electrode.[141]

C. Potentiometric Detection

Potentiometric sensors have not been frequently used in chromatography. Ion selective electrodes are often all too selective to be used for chromatographic detection. Manz et al.[142] compiled the literature in the last 20 years on the use of potentiometric cells as LC detectors and found 25 references altogether.

Only two groups appear to be rather active in using potentiometric electrodes to sense ions. Simon's group[117,118,142] has developed microelectrodes that can be used in open tubular column liquid chromatography as on-column detectors. Haddad and co-workers[143-149] reported on the use of metallic copper electrodes in a variety of applications. They noted that the potential of the copper electrode is dependent on the concentration of copper ion at the electrode surface, which in turn will be governed by the complexation properties of the eluent or the analyte. If the eluted solute forms a more stable complex with copper ions than the eluent ion, there will be a decrease in copper ion surface concentration and also a decrease in the potential. The events are just the reverse if the solute

forms the weaker complex with copper ions and if ion-exchange separation is used, where the sum of eluent and analyte ion concentrations is constant. Some applications are shown in Table 6.

Table 6
Some Ion Chromatographic Applications of Potentiometric Detection Using a Metallic Copper Electrode

Solute ions	Eluent	Ref.
Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}	Tartrate + diethylenetriamine	143
Organic acids	Phtalate, phosphate, or citrate	144
CN^-, Cl^-, Br^-, I^-, SCN^-	Tartrate	145
IO_3^-, BrO_3^-, ClO_3^-	Tartrate	145
NO_2^-, NO_3^-, SO_4^{2-}	Tartrate	145
Transition metal ions	Tartrate or citrate + ethylenediamine	146, 147
Ascorbate, hydrazine, hydroxylamine	Tartrate, citrate + ethylenediamine	148
Carboxylic acids	Orthophosphoric acid	149
Oxalate	Phtalate	149

IX. EC DETECTION OF CARBOHYDRATES — INNOVATIVE TECHNIQUES

Carbohydrates represent a biologically and industrially very important class of compounds which are difficult to detect in LC. UV and fluorescence detection cannot be applied directly. Carbohydrates are usually detected by refractive index measurement or after derivatization with UV absorbing or fluorescent tags.

Many carbohydrates, most notably the reducing sugars, are known to be easily oxidized chemically. This has created a challenge for many electroanalytical chemists to detect sugars by electrochemical means. Santos and Baldwin[150] collected 15 references on this subject from recent years. Direct oxidation of the carbohydrates on carbon working electrodes has very high overpotential and detection is not practical at the extreme positive potentials required. Oxidation of sugars occurs at much lower potentials, between −0.8 and +0.45 V on Pt, Au, or Ni. On the first two of these metals, adsorption processes quickly inactivated the electrode surface and rendered the usual technique of constant potential amperometric detection impossible. Ni is not plagued by this problem,[151] but electrochemists have always preferred electrodes made from the chemically more stable noble metals.

In a rather novel development, Johnson and co-workers[152-154] found that platinum electrodes can be used to detect carbohydrates by oxidation if the working Pt electrode is kept clean by periodical potential pulses. Originally, these workers cycled the electrode to three different potentials at the rate of about 1 Hz (Figure 20). A relatively positive potential (+800 mV) was used to clean or activate the electrode surface. A far more

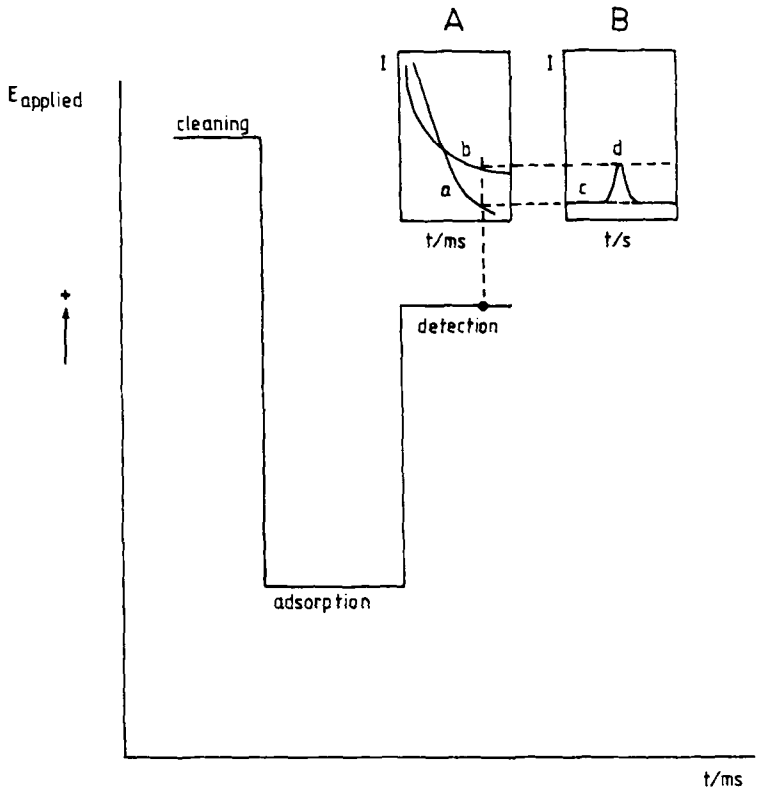


FIGURE 20. Triple step potential waveform in pulsed amperometric detection (PAD). (I) indicates measurement of current. (Inset A) Current transients during the detection phase: a = eluent, b = with analyte. (Inset B) Chromatogram: c = baseline, d = peak maximum.

negative potential was applied to promote adsorption of the analyte on the electrode. Finally, a suitably selected intermediate potential was used for detection. Since the detection potential was applied only for a fraction of a second, a transient current signal was observed. This transient depended on the concentration of analyte in the flowing stream of eluent. By reading the current transient always at precisely the same delay time, a quasi-amperometric signal is observed.

This method of cycling the electrode between detection, cleaning, and adsorption pulses has been called pulsed amperometric detection (PAD). Most notably it has been found that with suitable, experimentally selected potentials and timing, PAD can be used to detect many other organics. These are compounds which are thermodynamically predicted to be oxidizable at reasonably low potentials, but the oxidation is kinetically inhibited on platinum. The results obtained with a large variety of analytes have been summarized by Johnson[155] and Johnson et al.[156] Classes of successfully determined analytes include aliphatic alcohols, carbohydrates, aliphatic amines, amino acids, aminoglycoside antibiotics, sulfur compounds with a nonbonded pair of electrons on the S atom (e.g., thiourea), and some electroinactive adsorbates like CN^-. The utility of PAD has been verified on many real samples.

Sensitivity to the analytes depends on adsorption kinetics.

Sensitivity is usually highest in alkaline media. Peak height (i_p) vs. concentration (C_o) plots were linear for dilute samples. At higher concentrations, i_p^{-1} vs. C_o^{-1} plots were found to be useful. New, i.e., freshly polished, electrodes require 1 h or more to achieve a stable baseline. Later, the electrodes do not require polishing; in fact, polishing destroys the useful surface structure created by potential cycling.

In a commercial version of PAD,[157] use of the gold electrode has been recommended. Recently, Neuburger and Johnson[158] compared the performance of Pt and Au in PAD. It was concluded that PAD at Au electrodes has indeed the advantages of higher sensitivity and lower detection limits in comparison to Pt electrodes. For the LC-PAD determination of glucose, fructose, sorbitol, and sucrose, five times lower detection limits and increased linear region have been verified.

In yet another recent development, Neuburger and Johnson[159] report that a two-step potential wave form is sufficient for carbohydrate detection at an Au electrode. A separate adsorption step was found to be unnecessary. This innovation eliminates the need for specialized instrumentation. Commercial polarographs suitable for normal pulse polarography can be used with a slight modification.

A rather different approach for carbohydrate detection has been followed by Santos and Baldwin.[150] As noted before, oxidation of sugars is kinetically inhibited on carbon electrodes. This inhibition may be overcome by incorporating a suitable electron transfer catalyst into a carbon paste used for electrode preparation. Santos and Baldwin found that cobalt(II) phtalocyanine, a well-known electrocatalyst, satisfied the practical requirements of electrode stability, reproducibility, and ease of electrode fabrication.

The cobalt phtalocyanine-modified electrode did indeed oxidize carbohydrates at moderate positive potential (+0.39 V vs. an Ag/AgCl reference), but the signals decreased upon repeated injections, showing electrode fouling. This could be overcome by cycling the electrode potential between a cleaning (−0.3 V) and a detection (+ 0.39 V) level and sampling the current near the end of the detection period. This is virtually the same technique as that adopted recently by Neuburger and Johnson[159] for carbohydrate detection at a gold electrode. It remains to be clarified if the metal or the modified carbon paste materials offer more practical advantage.

The methods discussed here involved direct oxidation of carbohydrates at the working electrode of the detector cell. A useful alternative approach has been to oxidize the carbohydrates in a postcolumn reaction and to detect the reduced form of the oxidizing agent amperometrically. This is a theme where many variations are possible and each may offer specific advantages. Watanabe and Inoue[100] let the sugars reduce the copper(II) bis(phenantroline) complex at high temperature in alkaline solution and achieved a detection limit of 1 pmol (0.2 ng) for glucose. Good selectivity was provided by the low oxidation potential required (+0.075 V vs. Ag/AgCl). Honda et al.[97] used a different derivatizing agent, 2-cyanoacetamide. Detection limits were 20 times higher than those of Watanabe and Inoue but the method was universally applicable to all types of HPLC currently used for the analysis of carbohydrates. Marko-Varga[160] mixed the effluent from the chromatographic column with nicotinamide adenine dinucleotide (NAD^+) coenzyme buffer and passed it through a packed bed reactor containing immobilized glucose dehydrogenase enzyme. In the enzymatic oxidation of the carbohydrates, NAD^+ was reduced to NADH and the latter was detected by oxidation on a modified carbon electrode. The use of this system offering enzymatic selectivity was found to be justified in the analysis of a complex fermentation broth. Carbohydrate detection by indirect methods may also be based on conductivity[132,134] or potentiometric[161] measurement.

X. RECENT DEVELOPMENTS IN VOLTAMMETRIC EC DETECTORS

This section is short and far from comprehensive. The intention has been only to direct attention to some important and/or interesting subjects. More information may be gathered from recently published books[8,27] and reviews.[9,20]

A. Multiple Electrode Detection

Multiple electrode detection means the simultaneous use of two or more working electrodes. Typically, the working electrodes are kept at different potentials. This can be solved without the need to use multiple reference and auxiliary electrodes. Multiple electrode detection can be used to improve selectivity, detection limits, and peak identification. It can also extend the useful potential range of detectors based on carbon electrode material.

Multiple electrodes are not new in LCEC. Back in 1982, a review appeared[162] on this subject, and the idea of using multiple working electrodes can be traced back to the rotating ring-disk electrodes, a rather useful tool of electroanalytical chemists.

In the 1982 review, it was prognosticated that "multiple electrode detection in a variety of formats will help sustain the growth in LCEC applications during the next several years." This has indeed come true. Dual and other multiple electrodes are widely used and this is the reason why their advantages are reiterated here.

Possibilities for the arrangement of two working electrodes in a thin-layer cell are shown in Figure 21. In the parallel adjacent orientation, the operation of the two working electrodes is independent of each other. Being set to different working potentials, the electrodes record two simultaneous but different chromatograms. This is similar to UV detection at two different wavelengths. The advantage in analyte identification is obvious.

In the series dual-electrode arrangement, the downstream electrode is used to detect reaction products of the upstream electrode process. For example, the upstream electrode can be used to reduce an analyte. If the reduced form can be oxidized

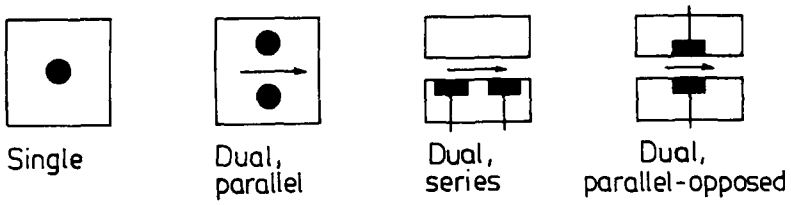


FIGURE 21. Scheme of single and dual electrode arrangements in a thin-layer cell. The arrows indicate the direction of flow.

on the downstream electrode, it can be measured there. This makes detection of directly not oxidizable analytes possible at the carbon electrode.

With parallel-opposed dual electrodes, current amplification can be achieved for reversibly behaved analytes. Analyte oxidized at the electrode set to the more positive potential is reduced at the other working electrode, then oxidized again on the first electrode, and so on. Clearly, flow rate and cell dimensions need to be such that the analyte has sufficient time to diffuse back and forth between the electrodes several times before it leaves the space between the electrodes. Current amplification is achieved because the same molecule is oxidized or reduced several times at the respective electrode.

It is also possible to use two completely different working electrode materials and geometries in a dual detection system, e.g., a thin-layer oxidative cell and a polarographic cell in a series arrangement. This allows simultaneous detection of oxidizable and reducible analytes. Tandem methods can go so far that a voltammetric detector is combined with a spectrophotometric one. Many references to such detector combinations are given by Slais.[116]

Multiple electrode detectors with a large number of working electrodes set at different potentials have not been frequently used. A set of amperometric electrodes in the parallel adjacent configuration would make the construction of three-dimensional time-potential-current chromatograms possible. When coulometric conditions prevail at the working electrodes, the same result can be achieved with the series electrode arrangement. This has been demonstrated recently.[41,42]

B. Potential Scanning

Single wavelength UV detectors, amperometric detectors, and most other detectors yield meaningful data only if the analytes are sufficiently separated on the chromatographic column. This places a heavy burden on the chromatographic method development. UV detectors with wavelength scanning or simultaneous multiwavelength measurement possibility produce a lot of extra information in the form of spectra recorded many times during a chromatographic run. Similar three-dimensional resolution may be achieved by frequently scanning the working electrode's potential between preset limits. Sufficient time resolution of the chromatovoltammograms requires that at least one scan is made per second. Assuming a 1-V range, this is equivalent to a 1-V/s scanning rate. At this scanning rate, charging currents, a kind of disturbing transient signal, become usually prohibitively high and detection limits are increased by orders of magnitude.

To overcome this problem, several techniques have been reported. The usual method in nonflow-through electrochemistry to get rid of charging currents has been the application of different potential pulse waveforms. After a potential jump, the charging current decays rapidly. Current is sampled only when the charging current has almost vanished. A variety of waveforms have also been used in LCEC,[163-170] but the detection limits could not be brought down to the amperometric level. Nevertheless, the selectivity advantage may be utilized also at higher concentrations.

A rather elegant way of potential scanning without the charging current disturbing the detection has been realized by Trubey and Nieman[171] and by Heineman and co-workers.[172-174] These investigators separated the scanning from the detection. They used a series dual-electrode thin-layer cell. The potential of the upstream electrode was scanned while the downstream detector electrode was maintained at a constant potential. Scans were made for instance from +0.3 to +1.3 V at the rate of 2.0 V s^{-1}. The detector electrode was set at −0.2 V. Analytes oxidized at the upstream electrode were reduced at the downstream electrode if the redox reaction was reversible. During any scan, the downstream electrode began to record a signal only when the upstream electrode reached a high enough potential to oxidize the analyte passing through the detector at the time of the scan. In this way, the current of the downstream electrode plotted against the potential of the upstream electrode gave fast voltammograms. A correction was made for the time delay between the two electrodes. With this technique, some electronic crosstalk is observed between the two electrodes, increasing the detection limit somewhat.

Trubey and Nieman[171] applied coulostatic scans, while the Heinemann group[172-174] utilized the conventional DC scan. Detection limits reported by the latter authors approached amperometric detection limits. The merits of potential scanning and of this particular technique were summarized as follows. The voltammetric data generated during the chromatographic run help to identify peaks. In case of co-elution, the individual peaks can be resolved voltammetrically. Recording the entire chromatovoltammogram in the computer allows post-run evaluation at optimum potentials, including the use of different potentials for each chromatographic peak. An additional ad-

vantage for the bioelectrochemist is that voltammograms of the individual components of a complicated mixture can also be obtained from minute amounts of sample.

Yet another method for potential scanning has been the use of microvoltammetric electrodes where the analytical Faradaic signal is enhanced when compared with the capacitive background. White et al.[114,175] have shown that an on-column detector for capillary LC made from carbon fiber may be scanned and useful data obtained. However, this technique is not yet relevant for normal-sized work.

C. Gradient Elution

When samples are encountered with many analytes of widely different affinities for the chromatographic packing used, gradient elution is the method of choice. There have been totally contradicting reports on the applicability of gradients with LCEC. Khaledi and Dorsey[176] undertook an interesting study to examine the situation. They analyzed earlier reports on the use of gradient elution with amperometric detection and noticed that the contradictions had been only apparent. There was always a drift of baseline observed, however, it was negligible when relatively concentrated samples were determined at low (e.g., +0.7 V) applied potential. On the contrary, when minute concentrations had to be detected at about +1.2 V, the baseline shift was intolerably high during the gradient run.

When the factors influencing the background current were searched, it was found that there were many: pH, viscosity, purity of reagents, cell design, and ionic strength (through the IR-drop), applied potential, electrode pretreatment, and the kind of organic solvent all could have an effect on the background current. For details, the original work should be studied. Comparison with some published works[41,177,178] using gradient elution appears to confirm the conclusions of Khaledi and Dorsey. These authors also suggest the use of micellar mobile phases because this can eliminate a great part of background shift.

D. Electrode Materials and Surfaces

The success of voltammetric EC detection depends to a very great extent on the working electrode. Voltammetric detection is based on an interfacial rate process and therefore it is inherently sensitive to surface contamination. Electrode fouling has deterred many from using electroanalytical devices. The dropping mercury electrode has had a unique role among electrodes. Its frequently renewing surface ensured consistent results.

The mercury electrode cannot be used, however, in a wide anodic range, i.e., for detection of oxidizable analytes. A number of solid electrodes, e.g., platinum, gold, and carbon, have a good anodic range but they are prone to fouling. Solid electrodes are also much more convenient to use in flow-through systems than the dropping mercury electrode.

A great deal of effort has been recently exerted to make using mercury electrodes more convenient (in the cathodic range) and to eliminate fouling at the solid electrodes used as anodes. Solid electrodes have been the subject of many surface studies.

One approach to eliminate fouling has been to exclude strongly adsorbing substances from the electrode surface. This can be done by casting thin protective films on the electrode surface. The films, e.g., cellulose acetate[179] or irradiated poly(ethylenimine),[180] exclude adsorbing materials like surfactants but allow the analytes and reaction products to pass. The drawback of this solution is that it does not help against the adsorption of analyte or reaction products, which can be rather serious.

Another approach has been to renew the electrode surface frequently. This can be done with mercury film electrodes on a solid substrate by stripping off and replating the mercury film after each analysis.[181] Solid surfaces may be renewed *in situ* by applying short laser pulses directed at the surfaces[182,183] or by using cleaning electrical pulses. The latter method has been rather successfully used by Johnson's group on platinum and by Rocklin and Pohl on gold. Their results have been reviewed in the section on carbohydrates detection. Wang and Lin[184] drastically decreased the passivation of glassy carbon due to important analytes (or their reaction products) like phenol, NADH, chlorpromazine, uric acid, and butylated hydroxyanisole. The cleaning pulses they used also prevented deactivation due to surfactants. The *in situ* renewal of the electrode surface appears to be more promising than most off-line electrochemical pretreatments.

However, electrodes can also be "pretreated" by chemically modifying their surface. This recent technique has enormous potential. The variety of techniques developed to date have been recently reviewed.[185] Chemical modification of the surface will hopefully make possible electrodes having predetermined selectivites, which are free from adsorptive and coating effects. This new and powerful tool has emerged paradoxically from the very problem it wants to solve: molecular or thin polymeric film coatings are deliberately applied to the electrode surface in a similar manner to that which occurs during unwanted adsorption or filming. Obviously, there is enormous variability in surface modification and this is the main strength of the new approach. Applications in LC have been rare, but this will certainly change.

Another method of electrode modification has been to use carbon pastes with incorporated modifying substances. The modifier exerts its effect mainly at the electrode/solution interface and therefore some authors regard this method as a special kind of surface modification. The work of Santos and Baldwin has been quoted in the section on carbohydrates detection. Their cobalt phtalocyanine-modified carbon paste electrode has also been used to detect oxalic acid and α-keto acids at significantly lower potentials than is possible with the unmodified carbon paste.[186] Wang and co-workers[187-189] have been very actively investigating the possibilities of using plant tissues and algae admixed to the carbon paste, while Matuszewski and Trojanowicz[190] could detect glucose at fairly

low positive potential with carbon paste containing glucose oxidase enzyme.

XI. FUTURE TRENDS

In this review, those areas of LCEC applications have been given detailed discussion where EC detection has already established itself as a useful tool. Further reports on applications in these areas will certainly come in great numbers, probably until the use of the EC detector is no longer exotic and it will only be mentioned in the experimental part of articles just as it is with UV detectors now. Much will probably be heard about pharmacokinetic applications since this fraction of the literature appears to be expanding most rapidly. Many other new applications are expected to be reported. EC detectors can be found now in many routine laboratories and will probably be tested as alternatives in those cases where UV detection presents a problem. In this way, new, perhaps not yet foreseen, applications may be discovered. It appears now that EC detection may require less sample pretreatment in many situations than UV or fluorescent detection and this will certainly be a great advantage.

Further work to improve electrode reliability is expected to be done. Surface modification may be one of the ways to do this but this technique may also produce not yet anticipated breakthroughs.

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