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LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

NEW APPLICATIONS IN ION CHROMATOGRAPHY AND REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

Electrochemical detection is shown to have the potential to be applied to an even wider range of techniques and conditions. It is useful in ion chromatography for detecting both electroactive and electroinactive ions. Carbon paste electrodes may be suitable for work at high concentrations of organic solvent and sample clean-up can sometimes be simplified without a substantial decrease in performance.

INTRODUCTION

Electrochemical detection (ED) in liquid chromatography (LC) has rapidly become very successful, particularly for the analysis of biological samples[1]. In a typical application, the determination of oxidizable analytes at a carbon working electrode, these detectors excel because their detection limits are low and the chromatograms are relatively clean owing to their selectivity. ED has also been used in ion chromatography to detect easily oxidized ions or ions that react with electrogenerated silver or mercury(II) ions[2,3].

In this work different ways of extending the range of applications of the LC–ED technique have been investigated: (1) introduction of ion chromatography with indirect ED; (2) use of short columns for routine high-performance liquid chromatography (HPLC) with ED; and (3) use of carbon paste-type electrode materials (a) in eluents with a high concentration of organic solvents, (b) for samples of biological origin with and without sample clean-up and (c) at relatively high positive potential.

Examples given include first reports on the LC–ED of three recently developed biologically active compounds.

EXPERIMENTAL AND RESULTS

Apparatus

For ion chromatographic work a Beckman (Fullerton, CA, U.S.A.) 114 M

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pump, a Labor MIM (Budapest, Hungary), OE 320 injector and a Biotronik (Maintal, F.R.G.) Type BT II AN 100 × 4.6 mm I.D. anion exchange column were used. The reversed-phase chromatographic system consisted of a Labor MIM Model LS 241 pump with pulse damper, an OE 320 injector, a Spherisorb 5-μm ODS column (250 × 4.6 mm I.D.), LiChrosorb RP-8,10 μm (Chrompack, Middelburg, The Netherlands), or an Ultrafast C_{18} 3 μm column (40 × 4.6 mm I.D.) (Perkin-Elmer, Norwalk, CT, U.S.A.). The ED 101-A electrochemical detector[4,5] used in both systems was constructed in our laboratory.

Chemicals

Methanol and all solid reagents were of analytical-reagent grade from Reanal (Budapest, Hungary). Water was doubly distilled from quartz glassware. Test compounds were kindly donated by Material (Chemical Industry Cooperative Society) (XAX-M) and EGIS (Budapest, Hungary) (Egyt 2509 and 3615). Serotonin, thyramine and the antioxidant (BHT) were Reanal products. Chemical names are as follows: XAX-M = bis(2,2,4-trimethyldihydro-6-quinolinoethane); BHT = 2,2-di-*tert.*-butyl-4-methylphenol; EGYT 3615 = 1-(4-chlorophenyl)-1-hydroxy-1,2,7a,8,9,10,11,11a-octahydroisoquinolino[1,2-*f*][1,2,4]triazine-5-ium-ethanesulfonate; EGYT 2509 = 5-(3-dimethylamino-2-methylpropyl)-5H,11H-dibenzo[*d*,*g*][1,3,6]dioxazocine.

Ion chromatography with indirect electrochemical detection

In recent years ion chromatography (IC) has become a widely used method[6–10]. Ions separated on a low-capacity ion-exchange column have been detected mainly by conductivity detectors, either directly after the separation column or after passage through a second ion-exchange device called a suppressor, which served to enhance the signal. IC systems can be also operated with detectors other than the conductivity type. Because UV and electrochemical detectors are most readily available, there have been many applications to IC. Many ions absorb UV light, particularly at the lower end of the UV range of analytical instruments, and there are also various ions that are electroactive on suitably chosen electrode materials. These ions can be detected directly in the eluate from the analytical column by UV or ED methods. Examples are nitrate for UV detection and cyanide and halide ions for ED on a silver working electrode. Direct UV and ED methods are obviously less general than conductometric detection methods. To overcome this drawback, indirect UV detection has been introduced[11]. This method is based on the observation that when sample ions are eluted from the analytical column there is a dip (a negative peak) in the eluent ion concentration. If the eluent ion is UV absorbing and the sample ion is not, then a negative peak can be observed. Of course, the baseline is very high because the eluent absorbs UV light. The success of the method therefore depends on the possibility of detecting small changes in a relatively large absorbance value.

Although there have been many interesting applications of ED in IC, the method has apparently not yet been applied as an indirect technique to IC. This technique relies on using an electroactive ion in the eluent and measuring the negative peak caused by the eluted sample ion. Hence ED can be used to detect electroinactive ions. Earlier methods based on ED for measuring electroinactive ions[12,13] depended on measuring the pH changes caused by eluted sample ions after a suppressor. In

contrast, the method presented here does not use suppression and is not related to changes in pH.

Fig. 1 shows the separation of chloride and nitrate by a salicylate eluent. Salicylate and salicylic acid are sufficiently electroactive on carbon electrode materials at potentials above *ca.* 900 mV *vs.* a silver–silver chloride electrode in 1 *M* potassium chloride solution. With this technique we have found detection limits below 1 ppm for chloride and nitrate with the particular system used. It should be noted that the column available to use had been designed for suppressed IC.

The main component of the eluent need not necessarily be electroactive for indirect ED. As electrochemical detectors have a very wide dynamic range, extending to much lower concentrations than typical eluent concentrations in IC, it is sufficient to add electroactive ions at some very low concentration to the eluent. Useful chromatograms were obtained with an eluent of 1 m*M* salicylate at working electrode potentials so low that salicylate is no longer electroactive. The peaks in Fig. 2 were due to 2,5-dihydroxybenzoic acid, which was added to the eluent in a 100-fold lower concentration than salicylate. 2,5-Dihydroxybenzoate is oxidized at much lower potentials than salicylate.

Apparently, all IC techniques designed to date have relied heavily on the low concentration of the eluent, otherwise detection would have become difficult. Also,

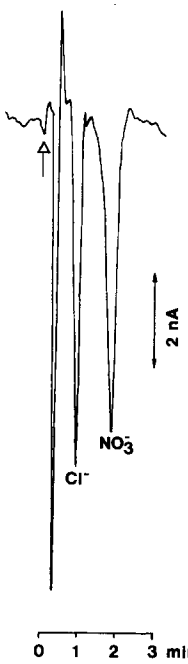

Fig. 1. Ion chromatogram with indirect ED. Sample, 5 ppm Cl^- and 10 ppm NO_3^-; flow-rate, 1.5 ml/min; injection volume, 100 µl; column, Biotronik BT II AN (100 × 4.6 mm I.D.) anion-exchange column; eluent, 0.1 m*M* salicylic acid–0.9 m*M* sodium salicylate; detector, carbon-paste electrode at +950 mV.

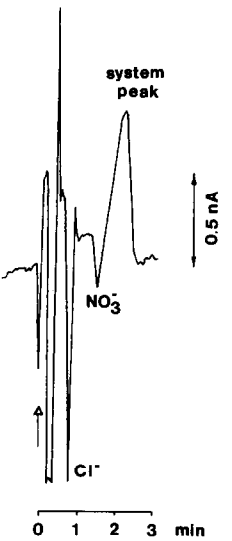

Fig. 2. IC with indirect ED and the main component of the eluent electroinactive. Sample, 2.5 ppm Cl^- and 2.5 ppm NO_3^-; eluent, 0.1 m*M* salicylic acid–0.9 m*M* sodium salicylate–10^{-5} *M* 2,5-dihydroxybenzoic acid; detector, carbon-paste electrode at +450 mV; other conditions as in Fig. 1.

the choice of eluent has been seriously limited by the detection technique. In suppressed IC of anions, the eluent anion must form a weakly dissociating molecule with the hydronium ion. In non-suppressed IC the eluent should have a low equivalent conductance or a suitable UV absorbance depending on the detection principle. If IC with indirect ED is used, the electroactive substance need not be the main component of the eluent and, therefore, the choice of the main component is much wider.

Electrochemical detection with short LC columns

As noted earlier, LC–ED has gained widespread use in the routine analysis of biological samples. Typically, a large number of samples have to be tested for analytes at very low concentrations. To increase the throughput and to lower the detection limit, it is reasonable to decrease the holdup volume of the column. In difficult separations this can be achieved by using microbore columns[14]. If the separation requirements are less stringent, short columns can be used. We have investigated this option in conjunction with ED. If short columns are used, chromatographic peaks are eluted faster and the detector response rate may become crucial. The detector response is sufficiently fast if the detector permits undistorted detection of five peaks in 30 s[15]. Fig. 3 shows that the detector used in this study meets this requirement. Electrochemical detectors are known to be sensitive to variations in flow-rate and consequently damping of pump pulsation and electronic filtering of pump noise are very important. We have investigated with short (40 mm) columns the effectiveness

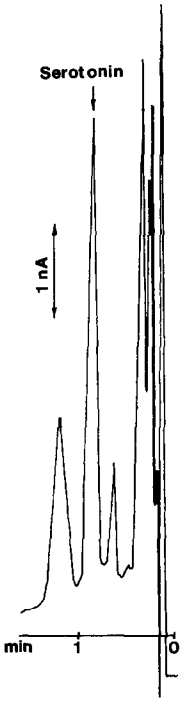

Fig. 3. LC–ED on a short column. Column, Ultrafast C_{18}, 3 μm (40 × 4.6 mm I.D.); eluent, 0.01 *M* KH_2PO_4 (pH 3); flow-rate, 0.5 cm^3/min; sample, meat pulp; $E = +0.8$ V *vs.* Ag/AgCl (1 *M* KCl).

of the electronic noise filter of the electrochemical detector. Two pumps were compared: a Waters 6000 A and a Labor MIM LS 241, both being used together with a mechanical pulse damper built in this laboratory. Table I shows the noise amplitude as a function of filter time constant. The filter type was a third-order Chebyshev low-pass.

The low-frequency noise of the Waters pump cannot be filtered electronically without seriously distorting the chromatographic signals. The spark noise of the other pump was efficiently swamped by the filter. This result shows that pumps suitable for UV detection are not necessarily suitable for ED. On the other hand, good electronic filtering may improve detection limits by an order of magnitude.

Electrochemical detection with a carbon paste electrode under extreme conditions

ED depends on a heterogeneous reaction: analyte is oxidized or reduced at the surface of the working electrode. This reaction may be severely influenced by the quality of the electrode surface. The latter depends on the electrode material and preparation and the eluent composition. The surface may also be altered by interaction with the analyte and with electroinactive components of the matrix, *e.g.*, adsorbing macromolecules.

A variety of materials are available for working electrodes[16]. In routine LC–ED carbon pastes and glassy carbon have been most frequently used. Carbon pastes appear to have lower detection limits while glassy carbon gives more reproducible results and is resistant to organic solvents. Glassy carbon needs careful cutting, polishing and embedding. Carbon paste can easily be made from inexpensive materials. Contaminated glassy carbon surfaces are repolished or cleaned by electrical pulses, while carbon paste electrodes need refilling with paste or renewal of the top layer.

In this study we investigated the silicone-based carbon paste electrodes of the ED 101-A detector with respect to interactions with the eluent and sample matrix.

High concentration of organic solvent. Organic modifiers in the eluent such as methanol and acetonitrile are known to disintegrate hydrocarbon-based carbon pastes. Addition of sodium lauryl sulphate to the paste has been suggested[17] to make it resistant to acetonitrile, but such pastes quickly disintegrate in water and partly aqueous eluents. Silicone polymer-based pastes withstand solvents to varying extents. We have found that the shelf-life of the silicone-based carbon paste electrodes of the ED-101 A exceeded one week when soaked in acetonitrile–aqueous eluents or pure acetonitrile. Three chromatographic examples are given below of the use of this electrode in eluents with high concentrations of organic modifier.

TABLE I

FILTERING OF NOISE CAUSED BY PUMP PULSATION

Pump	*Noise (pA) at time constant (s)*		
	0.1	*2*	*10*
Waters 6000A	50	50	50
Labor MIM LS 241	100	8.3	8.3

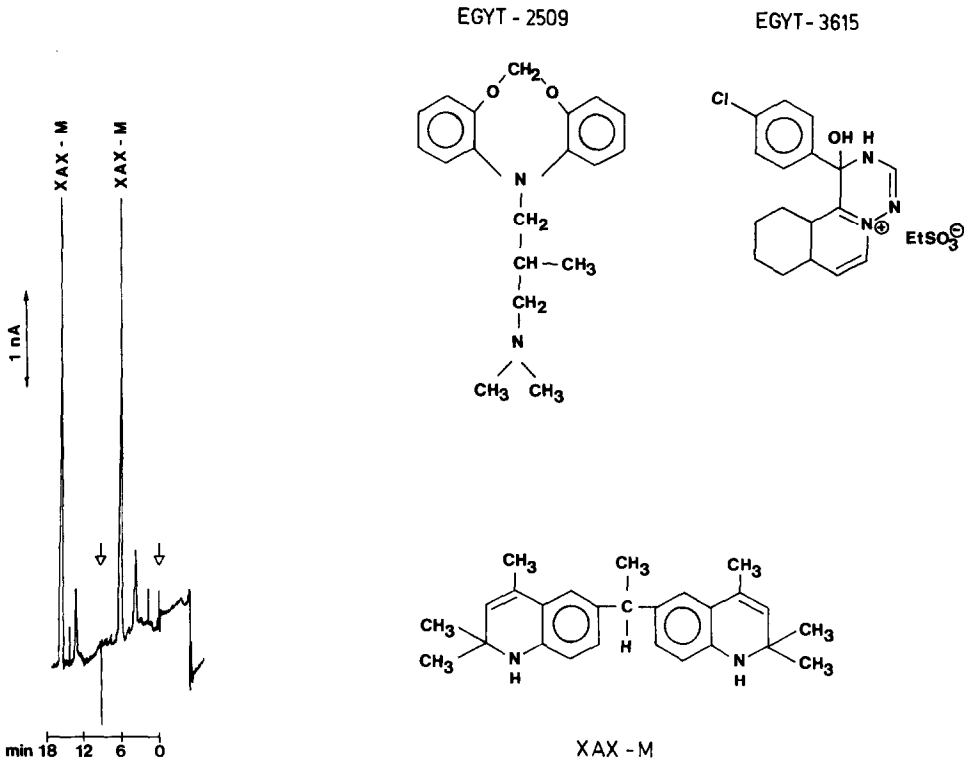

Fig. 4. Carbon-paste electrode in methanol-rich eluent. Column, Spherisorb ODS, 5 μm (250 × 4.6 mm I.D.); eluent, methanol–0.01 *M* KH_2PO_4 (90:10); flow-rate, 1.0 cm^3/min; $E = +650$ mV *vs.* Ag–AgCl (1 *M* KCl); sample, 7.5 pg of XAX-M.

Fig. 5. Structures of test compounds.

Fig. 4 shows the results for an XAX-M standard at very low concentration using an eluent containing 90% methanol.

The compound EGYT-2509 (Fig. 5) was determined in rat serum on LiChrosorb RP-18 after clean-up on a Merck Extrelut column. The eluent was 75% acetonitrile–25% 0.04 *M* KH_2PO_4 buffer containing 234 mg/l of sodium octyl sulphonate ion-pairing reagent. The detection limit for ED at 750 mV *vs.* Ag/AgCl (1 *M* KCl) reference was 2 ng/cm^3 and for UV detection at 282 nm it was 20 ng/cm^3.

Rat sera were cleaned on Waters Sep-Pak C_{18} before determination of EGYT 3615 (Fig. 5). The eluent was acetonitrile–methanol–water (160:120:60) and the column was LiChrosorb RP-8. The detection limit using ED was 1 ng/cm^3 and using UV detection (254 nm) it was 10 ng/cm^3.

The carbon paste had to be refilled after three days of continuous use in each case. This operation took about 5 min. Refilled electrodes had to be run in the system for about 20 min before injecting the next sample.

Simplified sample clean-up. The interaction between the sample matrix and the electrode surface may change the electrode sensitivity, background current and noise considerably. Therefore, sample clean-up is needed not only to remove substances

that give peaks overlapping the peaks of interest, but also to remove materials that are adsorbed on the electrode or otherwise cause changes in electrode behaviour. On the other hand, sample clean-up is often time consuming and tedious.

XAX-M is an antioxidant with two functional groups. In agriculture it is used as antiphoto-oxidant for vitamins in chicken feedstuffs, and also to decrease the amount of expensive ionophore-type antibiotics applied. The determinations of XAX-M in different animal tissues is therefore important. The interfering peaks of the matrix components in UV detection necessitate a very tedious clean-up procedure. It should also be considered that XAX-M is very sensitive to light.

It was of interest to establish whether ED is applicable to the determination of XAX-M in the presence of several endogenous compounds. First, ED was used for the determination of XAX-M in chicken tissues (Fig. 6) and second, serotonin was determined in an extract of meat pulp.

The XAX-M was extracted from the chicken tissues with acetonitrile[18] in an ultrasonic bath and the supernatant was filtered. During continuous routine work the electrode sensitivity changes by 1–5% per day and the background current remained virtually unchanged. The detection limit was 100 pg.

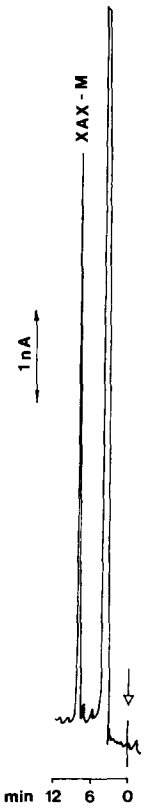

Fig. 6. Determination of XAX-M in chicken liver. Column, Spherisorb ODS 5 μm (250 × 4.6 mm I.D.); eluent, methanol–0.01 *M* KH_2PO_4 (85:15); flow-rate, 0.5 cm^3/min; sample, chicken liver, extracted with acetonitrile. XAX-M found, 20 pg per 20 μl.

In the other example, serotonin was extracted from a meat pulp sample with perchloric acid[18] and the filtrate was injected. A chromatogram is shown in Fig. 3. The detection limit was 1.5 pg. The electrode sensitivity and the background current typically changed by 5% and 2 nA, respectively, after three samples had been run.

Using a simplified clean-up, frequent checking of electrode sensitivity was necessary. After changing the carbon paste electrode calibration is necessary. In the first example above, determinations could be made without a sophisticated sample clean-up with reasonably frequent checking of sensitivity. In the second instance a more complicated clean-up is necessary.

High positive potential. Interactions between the eluent and the electrode and reactions of the electrode material itself may be responsible for the background current. The more positive the working electrode potential, the higher is the background current. Two implications of this are the increasing background noise and the increasing difficulty of compensating for the background current. The signal-to-noise ratio is expected to decrease at high positive potentials. This is obviously not true, however, if a compound can only be oxidized at high positive potentials. In this instance there will be an optimal, fairly high potential for the detection of the compound. For instance, the antioxidant BHT was determined in methanol–0.01 *M* KH_2PO_4 (80:20) at two potentials, 800 and 1200 mV *vs.* Ag/AgCl (1 *M* KCl). The background current was 6.2 and 15.0 nA, respectively, at these potentials. The linear dynamic ranges were, however, 0.5–500 ng and 0.08–500 ng, respectively.

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