Pulsed Electrochemical Detection of Sulfur-Containing Compounds Following Microbore Liquid Chromatography

George S. Owens and William R. LaCourse*, Department of Chemistry and Biochemistry University of Maryland Baltimore County 5401 Wilkens Avenue Baltimore, MD 21228 USA

* Corresponding author. Phone: (410) 455-2105, Fax: (410) 455-2608.

Pulsed Electrochemical Detection (PED) is a useful technique for detection of sulfur-containing compounds separated by microbore Liquid Chromatography (LC). In PED, both thiols and disulfides can be detected directly at a single gold electrode with limits of detection at the low picomole level. In this study, two common PED techniques, Pulsed Amperometric Detection (PAD) and Integrated Pulsed Amperometric Detection (IPAD), are investigated using model sulfur-containing compounds. Although both techniques can be used, results showed that IPAD resulted in better sensitivity and baseline stability than PAD. In addition, peak asymmetry (tailing), which is typically poor for sulfur-containing compounds in LC, was mitigated by a well-conditioned column and by use of acetate in the mobile phase.

Sulfur-containing compounds are ubiquitous in nature. These compounds include thiophosphate pesticides, antibacterial agents (e.g., penicillins and cephalosporins), and important biological agents and metabolites (e.g., cysteine, homocysteine, methionine, glutathione, coenzyme A, and biotin). Commonly, the reduced form of thiols is present with its oxidized (dimer) counterparts. The determination of sulfur-containing compounds in a variety of matrices is a problem of critical analytical significance.

Although organic (aliphatic) sulfur-containing compounds are readily separated from matrix interference by using reversed-phase chromatography, their detection is hindered by poor spectroscopic properties. Thus, alternate detection strategies for the direct detection of sulfur-containing compounds are of considerable importance. Electrochemical detection of sulfur compounds, with a Au-Hg amalgam electrode operated at a constant potential (DC amperometry), has been a popular, commercially available method. The Au-Hg electrode has been used in determination of amino acids (cysteine, homocysteine, methionine), peptides (glutathione, oxytocin, and vasopressin) and drugs, such as captropril (1-4). However, a single Au-Hg amalgam electrode is inadequate for detection of both thiols and disulfides, and a series dual-electrode configuration is required (5). Other approaches to DC amperometry include chemically modified electrodes (6), which are not at present commercially available.

An alternative to DC amperometry is pulsed electrochemical detection (PED). This technique involves applying a repetitive potential pulse sequence to a noble metal electrode, by which amperometric detection is combined with pulsed potential cleaning. As a technique for detection of sulfur-containing compounds, PED offers several advantages. Any compound in which the sulfur atom has an unshared pair of electrons is detectable by PED. Hence, a single electrode can be used to detect both thiols and disulfides. Since amine- and alcohol-based compounds require highly alkaline or acidic conditions for PED, selectivity for sulfur-containing compounds may be attained by using intermediate pH solutions. In addition, noble metal electrodes, required for PED, are robust and require a minimal amount of conditioning.

PED comprises a number of related techniques. The two most popular techniques are Pulsed Amperometric Detection (PAD) and Integrated Pulsed Amperometric Detection (IPAD). PED and its applications have been discussed in several review publications (7-10).

PAD uses the electrocatalytic properties of a noble metal electrode to drive analyte redox reactions, which are inhibited kinetically. However, constant potential operation of the noble metal elec-

trode results typically in fouling or deleterious oxide formation, which sharply attenuates the electrode response. To avoid this problem, a sequence of potential pulses is applied to maintain uniform and reproducible electrode activity. After the detection step, a pulse is applied at a potential sufficiently positive to cause anodic desorption of adsorbed species and formation of a metal oxide monolayer. The inert surface oxide is much less active than the "bare" metal, and a more negative potential pulse is subsequently applied to reduce the oxide monolayer to metal. This sequence of three potential pulses (i.e., detection, oxidation and reduction) is repeated continuously at a frequency of ca. 0.5 to 2 Hz.

In IPAD, the detection potential is ramped in a triangular waveform in the same manner as a cyclic voltammetric scan. Current is measured and integrated with respect to time to give a net charge for the detection cycle. Scan limits are set to encompass metal oxide formation and reduction. Charge balance considerations dictate that the charge for oxide formation is balanced by the charge for oxide reduction, which effectively cancels out signal from oxide formation. Since the oxide formation process is prone to variability due to surface conditions and local pH changes, the coulometric rejection of its formation signal by IPAD is advantageous. As with PAD, the detection step is followed by alternated anodic and cathodic polarizations. IPAD is most useful for amines and sulfur compounds; in that, the redox activity of amine and sulfur compounds is concomitant with surface oxide formation. As a consequence, considerable metal oxide formation background signal is a part of the analytical signal.

Pulsed Electrochemical Detection (PED) has been applied previously to the determination of sulfur-containing compounds, which include thiourea (11,12), penicillins (13), insecticides (14), and amino acids (15). These applications have focused on the detection properties of these compounds under highly alkaline or acidic conditions. Recently, LaCourse has published the first application of PED for sulfur-containing compounds using microbore chromatography (16).

Microbore LC offers the advantages of low solvent consumption and a smaller sample size requirement. In order to avoid the effects of excessive band-broadening and poor detection limits in microbore LC, extra column effects must be minimized in proportion to the smaller volume of eluting solute bands. Electrochemical detection is an attractive means of attaining small detector cell volumes, because electrochemical reactions require only a surface, rather than a volume, and electrodes are easily miniaturized.

In this paper, PED is used in conjunction with microbore LC for the determination of sulfur-containing compounds. Relevant aspects of sulfur compound electrochemistry will be used as guidelines for optimization of PED waveforms for electrochemical detection. Analytical figures of merit are given for several chromatographic separations.

EXPERIMENTAL

Apparatus

Voltammetric data were obtained at gold rotated disc electrodes (RDE) using a Model AFMSRX rotator and a Model AFRDE4 potentiostat (Pine Instrument Co., Grove City, PA). Data acquisition and potentiostat control were accomplished with a 486/33 MHz IBM-compatible computer (Entre Computer Systems, Columbia, MD) interfaced via a DAS-1601 high-speed AD/DA expansion board (Keithley Data Acquisition, Taunton, MA). Pulsed voltammetric waveforms were generated with ASYST scientific software (Asyst Software Technologies, Inc., Rochester, NY). For cyclic voltammetric data, potential ramps were generated by the potentiostat.

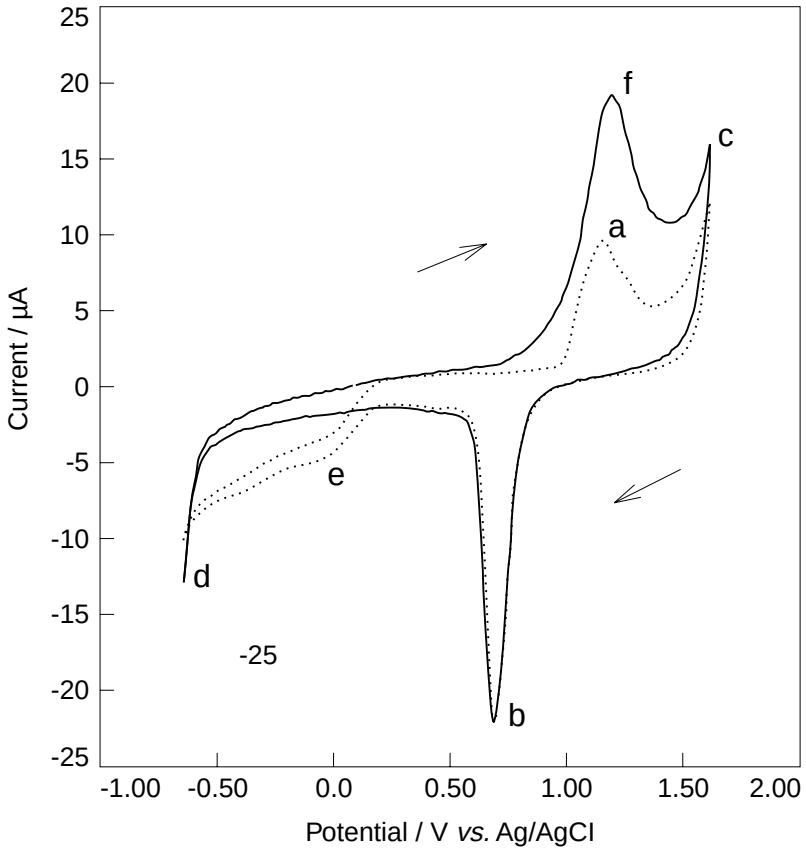
A gold RDE (Pine) of ca. 1.0 mm diameter was used for all cyclic voltammetry (CV) and pulsed voltammetry (PV) experiments unless otherwise noted. For these experiments a Pt auxiliary electrode and a Ag/AgCl reference electrode (Model 13-602-45, Fisher Scientific, Pittsburgh, PA) were used. All electrode potentials were reported *vs.* Ag/AgCl. The electrochemical cell (ca. 125 mL) was constructed from Pyrex glass with two side arms separated from the cell body by fine glass frits.

Microbore LC instrumentation consisted of a Model 4500 solvent delivery pump (Waters Chromatography Division, Millipore Corp, Milford, MA) coupled to a LO-Pulse pulse dampener (Rainin Instrument Co., Woburn, MA) and a flow splitter system (Bioanalytical Systems, Inc. (BAS), West Lafayette, IN). The flow splitter system consisted of an "in-line" filter and a stainless-steel tee, one arm of which was connected to a Phase II ODS chromatography column (3.2 mm x 100 mm) via stainless steel tubing, and the other arm connected to a Model 7520 injector valve with a fixed 0.5 µL sample loop (Rheodyne Corp., Cotati, CA). The flow was nominally split in a 10:1 ratio, in order to achieve flow rates of less than 90 µL/min. required by microbore chromatography. Separations were performed on a 100 mm x 1 mm, C_{18} (3 µm packing) "Uni-Jet" column (BAS) which was mounted directly to the injector valve to minimize dead volume. The work reported here was performed on more than one column.

PED was accomplished using software-generated (ASYST) PAD and IPAD waveforms with the computer-controlled potentiostat described earlier at a flow through electrochemical cell of a wall-jet type configuration. A detailed description of the electrochemical cell has been described elsewhere (16). The distance between the end of the

F1

Voltammetric response for DTE at a 1 mm Au RDE in 95% 0.1 M phosphate buffer (pH 3)/ 5% MeCN. Rotation speed: 900 RPM. Scan rate 200 mV s^{-1}. Solutions: DTE, 100 µM; and residual.



PEEK tubing and the working electrode was ca. 0.06 mm. The chromatography column was connected to the detection cell through the 0.005" PEEK tubing. In the compartment around the working electrode/PEEK extension tube containing the supporting electrolyte, a platinum auxiliary electrode was placed along with a Model MF-2021 Ag/AgCl reference electrode (BAS). The entire cell assembly was placed in a grounded metal cabinet, which functioned as a Faraday cage.

Reagents

Sulfur compounds dithioerythritol (DTE), trans-1,2-dithiane-4,5-diol (DTH), cystamine, 2-aminoethanethiol (AET), reduced glutathione (GSH) and oxidized glutathione (GSSG) were reagent grade (Aldrich Chemical Co., Milwaukee, WI). Reduced and oxidized cys-gly and -glu-cys were obtained from Sigma Chemical Co. (St. Louis, MO). All sulfur compounds were used as received without further purification. All solutions were prepared from reagent grade chemicals (Fisher Scientific, Springfield, NJ). Acetonitrile (MeCN) was "Optima" grade (Fisher). Water was purified using an IonPure reverse-osmosis system coupled with multi-tank/ultraviolet/ultrafiltration (U.S. Filter/IONPURE, Lowell, MA).

Procedure

Pulsed voltammetry (PV) scans were generated by incrementing one component of the PAD waveform and measuring resulting current. The objective of PV studies was to optimize PAD waveform parameters, similarly to a previous study with carbohydrates (17). PV scans were performed in a blank solution, then repeated immediately upon addition of the analyte of interest. Background-corrected voltammograms were produced by subtracting the residual response from the analyte response.

For the IPAD waveform, the detection step parameters were set so that the start potential was lower than the AuO reduction peak, and the peak potential was near the AuO formation peak. In addition, the net integrated current (i.e., charge) of the background was near zero. A fast-scan cyclic staircase voltammetry program was developed to determine these peak potentials. Cleaning pulses were set at potentials approximating those for PAD.

Microbore LC work was performed with mobile phases comprising an aqueous component and an acetonitrile organic modifier. The aqueous component was buffered to produce one of three pH values: pH 3, 0.1M phosphate buffer; pH 4.75, 0.1M acetate; and pH 6.5, 0.1 M phosphate. The pH range was selected to be compatible with silica-based chromatographic packings.

The relationship used for number of theoretical plates (N) is as follows (18):

$$N = 47.1 \left(t_r / w_{0.1} \right)^2 / \left(A / B + 1.25 \right)$$

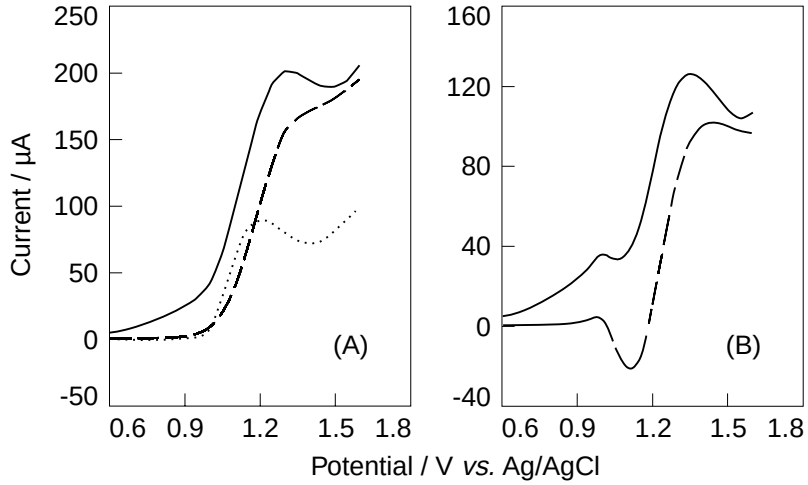
where, t_r is retention time, $w_{0.1}$ is peak width at 10% peak height, and A/B is the peak asymmetry factor obtained at 10% peak height.

RESULTS AND DISCUSSION

Electrochemical characterization was performed with model thiols and corresponding disulfides. Compounds were selected to meet the criteria of simplicity, solubility in mobile phase, minimal toxicity, low cost, predominantly aliphatic character, and availability of an unshared pair of electrons on the sulfur atom in the compound.

Cyclic Voltammetry

F1 shows the current-potential (I-E) plot for DTE, a thiol, in 0.10 M phosphate buffer, pH 3/MeCN (95/5; v/v). The residual response shows an anodic peak at ca. +1.15 V (wave **a**) during the forward scan due to the formation of surface oxide, or $Au(OH)_{x<<1} \rightarrow Au(OH)_{x\approx1}$



F2

Pulsed-voltammetric response, as a function of E_{det}, for DTE and GSSG at a 3 mm Au RDE in 95% 0.1 M phosphate buffer (pH3)/5% MeCN. Rotation speed: 900 rpm. Voltammograms, without background correction (A) are contrasted with background corrected response (B). Solutions: 200 µM; DTE, 100 µM; and residual.

→ AuO. On the reverse scan, a cathodic peak at ca. +0.68 V (wave **b**) corresponds to cathodic dissolution of the surface oxide formed on the forward scan. Solvent breakdown occurs at ca. +1.5 V (wave **c**) and ca. -0.6 V (wave **d**) resulting in O_2 generation and H_2 formation, respectively. Dissolved O_2 reduction occurs on both the forward and reverse scans commencing at ca. +0.20 V (wave **e**).

Addition of DTE results in an anodic peak which commences at ca. +0.4 V and peaks at ca. +1.15 V (wave **f**). It is conjectured that -SH is being oxidized to SO_3^-(19) with surface oxides facilitating the transfer of oxygen to the analyte. Hence, the DTE wave is found to be coincident with AuO formation, and this detection is denoted as being oxide-catalyzed. Adsorption of DTE to the Au electrode is indicated by the virtually complete attenuation of the reduction wave of dissolved O_2 due to blockage of sites on the electrode surface due to adsorbed DTE. The oxidation of DTH is similar to that of DTE.

The oxidation of DTE is typical of all sulfur-containing compounds, except for the following differences. The location of the anodic peak for sulfur oxidation is shifted to more positive potentials by ca. 0.10 V for the redox couples GSH/GSSG and AET/cystamine. In addition, the AuO reduction peak becomes broader, and its peak height decreased with AET/cystamine and GSSG/GSH from that of DTE/DTH. However, total peak area (proportional to total charge for reduction) did not change significantly between all the compounds tested, which indicates that it is not the extent of oxide formation on the forward scan which has been changed. Hence, this behavior is speculated as either a localized change in pH or slower electron-transfer kinetics due to the presence of a surface species related to analyte adsorption.

The percent organic modifier (i.e., MeCN) in the mobile phase does not affect the voltammetric response in the range of 5 to 40%. The pH of the test solutions had the largest effect on the location of CV peaks. Since surface oxide formation is pH-dependent by ca. -60 mV per pH unit, the anodic oxidation peaks of all PED-active compounds will shift similarly. The effect of pH has been described previously (20).

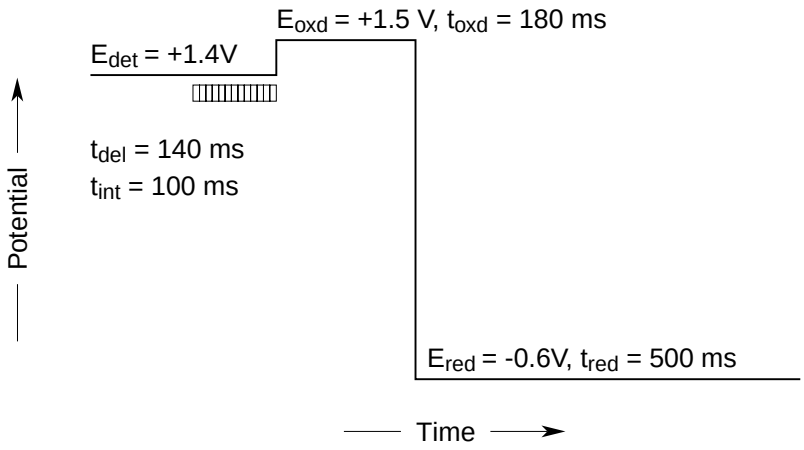
Pulsed Voltammetry

F2A shows pulsed voltammograms of detection potential (I *vs.* E_{det}) for DTE, GSSG, and 95% 0.01 M phosphate buffer (pH 3)/5% MeCN. The residual shows an anodic wave corresponding to oxide formation at E > ca. +0.90 V. The most substantial difference between sulfur compounds is the extent to which the analyte suppresses the onset of oxide formation. Note in ***F2A*** that the PV response for GSSG commences at potentials more positive then the onset of oxide formation in the residual. Hence, the "true" residual is not known. ***F2B*** shows the background-corrected responses of GSSG and DTE. Subtraction of the residual in the absence of analyte from the analyte response results in a net negative response in the region of ca.+0.90 to ca. +1.20 V for strongly adsorbed analytes (e.g., GSSG). Although the negative response is artificially created via subtraction, the effect can be directly correlated to peak height in LC. Negative peaks in HPLC-PAD have also been observed for penicillins, and they can be exploited as an indirect detection mode (13). On the other hand, weakly-adsorbed analytes (e.g., DTE, DTH, AET, and cystamine) show less shifting of anodic response, and consequently, no negative net response is observed. For all the compounds in this paper, the peak background-corrected response occurs in the range of ca. +1.25 to ca. +1.45 V.

All PAD waveform parameters were optimized by PV for each of the model compounds. The signal for pulsed voltammograms of delay time (t_{del}) and integration time (t_{int}) increased substantially at very low values, but the background noise increased disproportionately to the signal. Plots of the signal-to-noise ratio (S/N) were used to select the optimal values for t_{del} = 140 ms and t_{int} = 100 ms. The positive potential pulse was chosen to give maximum oxide coverage, which directly correlates with cleaning of the electrode surface. During the negative potential step, both oxide dissolution and preadsorption of the analyte occurs. The response for sulfur-containing compounds showed a linear upward trend of background-corrected current with the length of time (t_{red}) that E_{red} is applied. Current increased linearly by a factor of 2.5 in the t_{red} interval of 100 to 1000 ms. This behavior is consistent with a time-dependent adsorption isotherm for sulfur compounds (9,10). In selecting optimal parame-

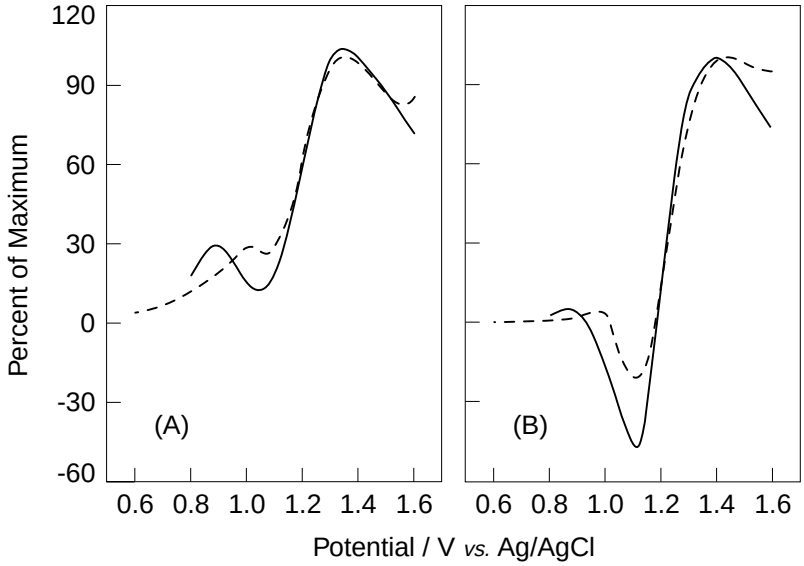
F3

Optimal PAD waveform.



F4

Comparison of pulsed voltammetry response with LC peak heights, on a normalized basis, for (A) DTE and (B) GSSG. (—) LC data; (– – –) pulsed voltammetry data. Conditions for PV: See F2. LC conditions: C18 1 mm UniCell column; 65 µL/min.; 0.5 µL injection volume, and optimized PAD waveform. Analyte concentrations: DTE, 100 µM/7.77 ng injected; GSSG, 200 µM/61.2 ng injected.



T1

Quantitative parameters of model sulfur compounds at a gold electrode in 95% 0.1 M phosphate buffer (pH 3)/5% MeCN by PAD.

Compound	Linear Range nA = *a*(pmole) + *b*				Repeatability %RSD (pmole, n)
	LOD, pmol	a	b	R^2	
DTE	2	42	-40	0.9995	2.2 (20,6)
DTH	3	26	-15.6	0.9953	2.8 (30,6)
AET	1	68	-4.8	0.9984	3.0 (10,6)
Cystamine	0.5	118	86	0.9978	3.0 (5,6)

ters for PAD operation, the overall cycle time was kept to a minimum to increase the frequency of the waveform in order to maintain peak integrity of the narrow solute bandwidths. Thus, t_{red} was set at a maximal 500 ms, and a t_{del} value of 140 ms was used with a slight sacrifice in S/N. E_{det} of +1.4 V was determined to be optimal for the widest variety of sulfur compounds. The optimal waveform used for microbore LC, in a pH 3 buffer/5% MeCN solution, is shown in ***F3***.

HPLC - PAD

For similar electrode material and hydrodynamics, the results predicted from PV experiments are comparable to LC-PAD peak height trends for (A) DTE and (B) GSSG; see ***F4***. Hence, the optimized parameters found using PV are readily transferable to LC-PAD.

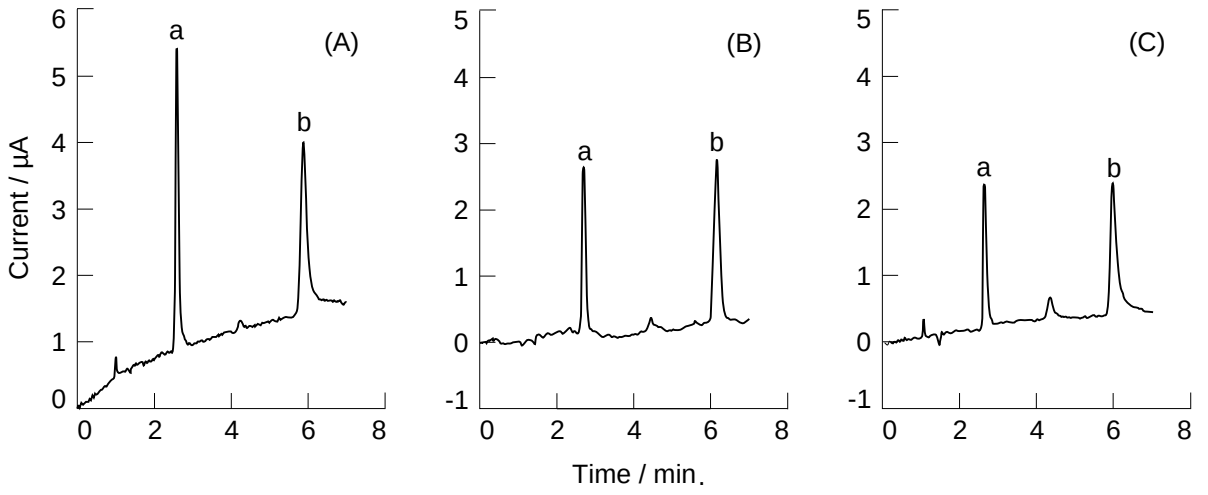
Analytical figures of merit for PAD detection in a 0.1 M phosphate buffer (pH 3)/5% acetonitrile mobile phase are presented in ***T1***. Limits of detection are on the order of 0.5 to 3 pmol injected. Linearity of the calibration curve is two orders of magnitude, which is typical of PED for strongly adsorbed compounds. It may be noted that calibration curve slopes for analytes cystamine and AET are very high. This is because these species, with charged amine groups, are retained very weakly, and band-broadening minimally decreases peak height. Injection to injection reproducibility is reasonable, yielding 2 to 3 % RSD values.

Sample chromatograms for mobile phases at (A) pH 3, (B) pH 4.75, and (C) pH 6.5 are shown in ***F5*** for (a) DTE and (b) DTH. Mobile phase pH did not greatly affect retention times of either compound. Peak response decreases with increasing pH for both analytes. Enhanced on-column oxidation of DTE at pH > 3 may explain its lower response. An unidentified peak at ca. 4.2 min. is attributed to the product of on-line DTE oxidation.

The peaks for (a) DTE and (b) DTH in ***F5*** are asymmetrical due to tailing. This effect has been noted before, and it is attributed to analyte interaction with metal ions in the chromatographic system (21). Peak asymmetry factors were quantitated with the IPAD waveform; see above. DTH showed more tailing than DTE. In the case of the pH 4.75 acetate/MeCN mobile phase, it is apparent that peak shape for DTH is distinctly improved compared to pH 3 and 6.5, which both used phosphate buffers. This observation suggests a possible beneficial effect on peak shape from either the use of acetate as a mobile phase component or the higher buffer capacity of the pH 4.75 ace-

F5

Comparison of LC response at various values of pH of the mobile phase; (A) pH 3, (B) pH 4.75 , (C) pH 6.5. Chromatography conditions are as in F4. The PAD waveforms for (A) and (B) were the same as for F3, while for pH 6.5 (C), all potentials were decreased by 0.20 V (i.e., E_{det} = 1.20V). Peaks: DTE, 15.4 ng, DTHN, 15.2 ng.



T2

Peak asymmetry and number of theoretical plates obtained from a BAS UniJet column and two representative sulfur compounds. Standard deviations, based upon five measurements, are in parentheses.

	Peak Asymmetry Factor		N	
Condition	DTE	DTHN	DTE	DTHN
pH 3	1.16 (0.05)	1.65 (0.03)	3370 (160)	4570 (100)
pH 4.75	1.27 (0.09)	1.30 (0.03)	3370 (280)	6190 (280)

tate buffer. The addition of acetate ion to the phosphate buffer adjusted to pH 3 also gave distinctly improved peak symmetry for DTH. Asymmetry factors were in the range of 1.1 to 1.2.

HPLC - IPAD

F6 shows chromatograms for a mixture of DTE and DTH using (A) PAD and (B) IPAD. The superior baseline stability and enhanced S/N of IPAD is clearly evident from ***F6B***. Limits of detection for these compounds are 1 and 2 pmole for DTE and DTH, respectively. The IPAD waveform, optimized for pH 3, is presented in ***F7***. For pH 4.75, all potentials were decreased by 0.10 V to accommodate pH-dependent shifting of the surface oxide background.

Since measurement of theoretical plates is significantly dependent on the peak asymmetry factor, which is in turn much more reliably obtained with a very flat baseline, the IPAD waveform is better suited for measurements of this parameter. ***T2*** lists N and peak asymmetry factors at pH 3 and 4.75 for DTE and DTH. There was little difference observed between the results for the IPAD versus the PAD waveforms, (using pH 3 mobile phase) except that variability in the data for IPAD was lower than for PAD.

As discussed earlier, DTH shows more tailing in pH 3 mobile phase than pH 4.75 mobile phase. This effect is statistically significant at the 95% confidence level. The difference in asymmetry factor for DTE at pH 3 and 4.75 is not statistically significant at a 95% confidence level. The improved peak asymmetry of DTH in acetate buffer is also reflected in the higher number of plates. When a fresh column was used, much greater peak tailing was observed than with the used column. For instance, asymmetry factors for DTH were 1.81 and 3.18 in acetate (pH 4.75) and phosphate (pH 3) mobile phases, respectively. This observation suggests that conditioning of new columns is necessary.

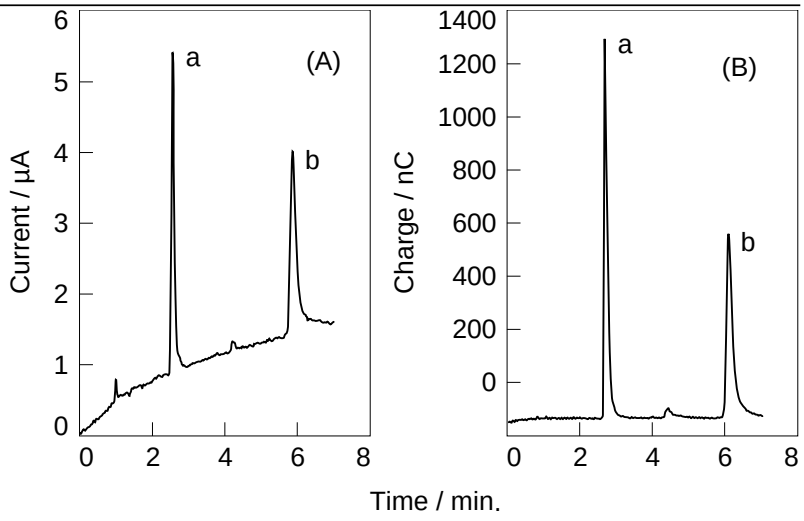
As an example of application of PED to biologically relevant samples, ***F8*** shows the separation and detection of a mixture of glutathione and glutathione fragments. These compounds have been mentioned as major and minor components of bacteria, plants, fungi, and animal tissue (22). The high sensitivity and selectivity at mildly acidic pHs for sulfur-containing compounds results in simpler chromatograms and less sample preparation.

CONCLUSION

Pulsed electrochemical detection allows for the *direct* and *sensitive* determination of thiols and disulfides at a single electrode. Results obtained for model sulfur compounds show limits of detection on the order of 0.5 to 3 pmol, linearity of two orders of magnitude, and reproducibility on the order of 2 to 3% RSD. The detectability of sulfur compounds over a wide range of pH conditions affords *compatibility* with reversed phase chromatography, which represents the separation mode of choice for sulfur-containing, aliphatic compounds. The high *selectivity* of PED for sulfur moieties under mildly acidic conditions reduces sample preparation and produces simpler chromatograms of complex mixtures. Although both PAD and IPAD are applicable to the detection of sulfur-containing compounds, IPAD results in higher sensitivity and better chromatographic stability.

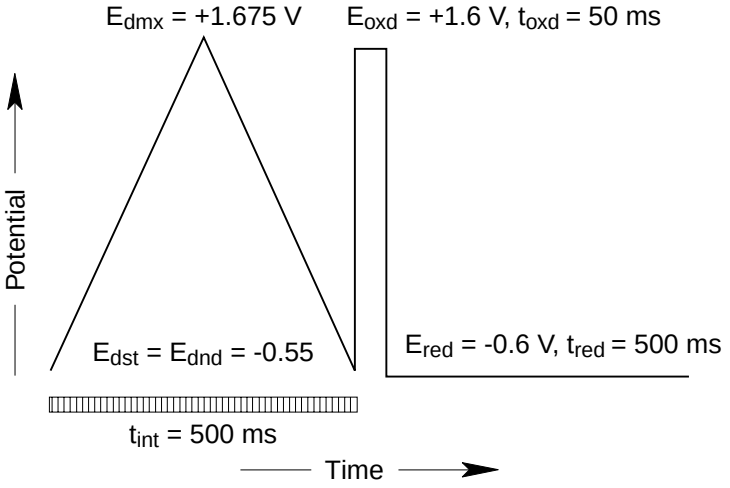
F6

Comparison of (A) PAD and (B) IPAD following the microbore separation of DTE, 15.4 ng, and DTHN, 15.2 ng. LC conditions are the same as in F4.



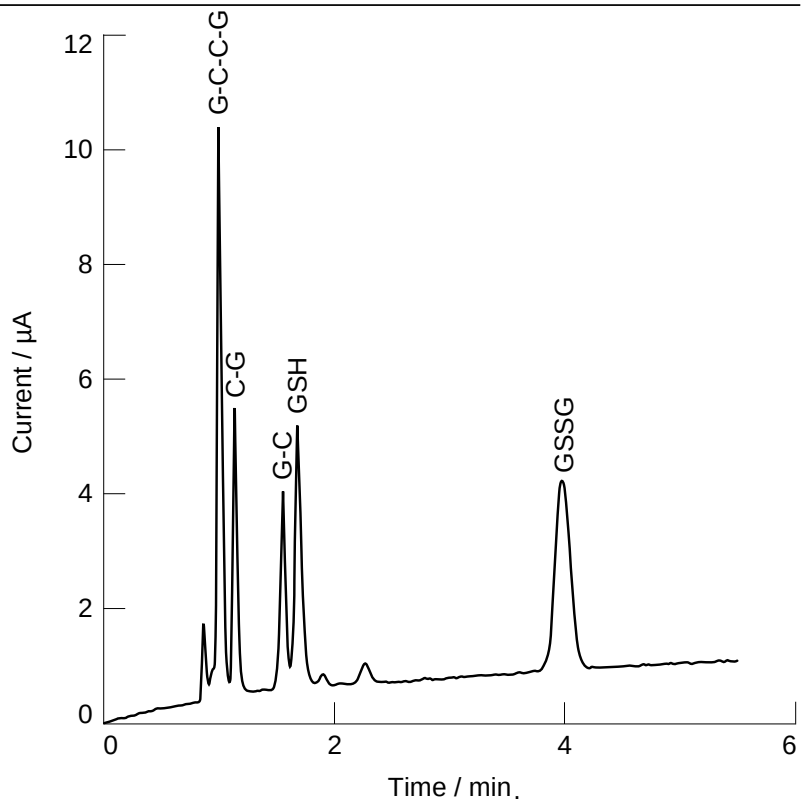
F7

Optimal IPAD waveform.



F8

Microbore LC separation of bioactive compounds: glutathione and glutathione fragments. Conditions are as in F4 except a 99.6% 0.1 M phosphate buffer (pH 3)/ 0.4% MeCN mobile phase were used, and flow rate was 76 µl/min. Peaks (all at 200 µM and 0.5 µL injection volume): (a) oxidized Cys-Gly, 35.4 ng; (b) Cys-Gly, 17.8 ng; (c) Glu-Cys, 18.5 ng; (d) GSH, 30.7 ng; and (e) GSSG, 61.2ng.



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