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Journal of Chromatography A, 1066 (2005) 133–142
JOURNAL OF CHROMATOGRAPHY A
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A pulsed potential waveform displaying enhanced detection capabilities towards sulfur-containing compounds at a gold working electrode

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Received 28 October 2004; received in revised form 23 December 2004; accepted 27 January 2005

Abstract

Pulsed electrochemical detection of sulfur-containing compounds was successfully investigated by applying a four-step potential waveform at a gold working electrode. This potential waveform called APAD, which stands for activated pulsed amperometric detection, is composed of an activation potential step added to a conventional three-step potential waveform. A key advantage of the APAD at the Au electrode is the ability to enhance sensitivity through the use of a short potential pulse (E_{ACT} = +750 mV versus Ag|AgCl and $t_{ACT} \approx 90$ ms) during which the formation of redox active species (presumably $OH^{\bullet}$) are able to efficiently oxidize organosulfur compounds. The APAD waveform parameters were optimized to maximize the signal-to-noise ratio (S/N) and successfully applied for the sensitive detection of lipoic acid, biotin, iminobiotin, methionine, cystine, cysteine, homocysteine, homocystine, *N*-acetylcysteine and glutathione, following their separations by high-performance anion-exchange chromatography (HPAEC) using alkaline mobile phases. The detection limits (S/N = 3, 10 μL injected) ranged from 0.3 for cysteine (400 pg) to 0.02 μmol/L for biotin (50 pg) and methionine (30 pg). The response of sulfur-, amine- and alcohol-based compounds was compared by using four selected pulsed potential waveforms. It was found that the APAD exhibits excellent sensitivity for thiocompounds outperforming all other pulsed potential waveforms. Ratios of the peak areas for APAD and the six-step potential integrated waveform increased from 3.2 ± 0.4 to 13.5 ± 0.6 for lipoic acid and biotin, respectively.

Keywords: Pulsed amperometric detection; Gold electrode; Anodic detection; Sulfur-containing compounds; Biotin; Lipoic acid

1. Introduction

Electrochemical detection has developed into a mature detection technique for determinations of polar aliphatic compounds, following flow injection and especially liquid chromatographic separations. Of the various electrochemical methods, constant potential detection is the simplest and works well for catechols, phenols, aminophenols and catecholamines [1–3]. However, inconsistent response is usually observed for polar organic compounds due to electrode fouling from sample matrixes and/or analyte reaction products at noble metal electrodes, such as platinum and gold [4,5]. Moreover, these organic molecules lack a strong chromophore or fluorophore, thus are barely detected by conventional photometric detection. To get a reproducible electrochemical signal over time, the electrode surface needs to be cleaned periodically by mechanical polishing or by a potential pulse. In pulsed electrochemical detection (PED) the electrode surface is renewed within a pulsing potential waveform that continually cleans and reactivates the working electrode. This detection scheme has become a powerful tool for chemical, clinical, pharmaceutical, and environmental sciences, owing to its excellent sensitivity and moderate selectivity toward electroactive compounds [6–9]. The most basic pulsed amperometry employs a three-step waveform incorporating potential excursions to produce a pristine electrode surface prior to each current measurement. Developed by Jonhson and co-workers [10–13], pulsed amperometric detection, PAD (see entry I in Table 1) was originally applied for carbohydrate detection at Au electrodes in alkaline solutions, following their separations by high-performance anion-exchange chromatography (HPAEC) [14–19].

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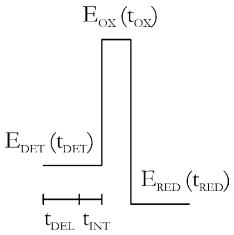
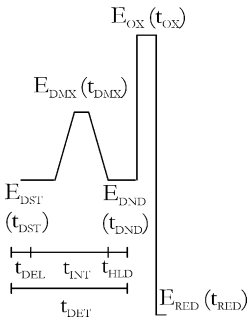
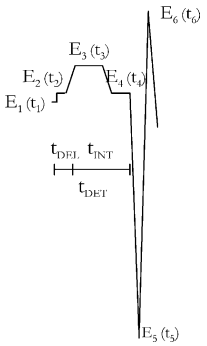
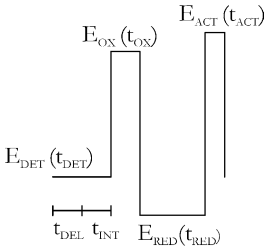
doi:10.1016/j.chroma.2005.01.076

Table 1
Potential–time parameters of pulsed amperometric detection waveforms

Waveform description		Potential (mV vs. Ag|AgCl)		Time (ms)			
I [15]		E_{DET}	+50	t_{DET}	440	t_{DEL} t_{INT}	240 200
		E_{OX}	+650	t_{OX}	180		
		E_{RED}	−220	t_{RED}	380		
II [31]		E_{DST}	−100	t_{DST}	200	t_{DET}	700
		E_{DMX}	+350	t_{DMX}	100	t_{DEL}	100
		E_{DND}	−100	t_{DND}	200	t_{INT}	500
		E_{OX}	+900	t_{OX}	190	t_{HLD}	100
		E_{RED}	−900	t_{RED}	90		
III [32]		E_1	−200	t_1	40	t_{DET}	560
		E_2	−50	t_2	60	t_{DEL}	110
		E_3	280	t_3	290	t_{INT}	450
		E_4	−50	t_4	140		
		E_5	−2000	t_5	10		
		E_6	+600	t_6	10		
IV		E_{DET}	+50	t_{DET}	420	t_{DEL} t_{INT}	220 200
		E_{OX}	+650	t_{OX}	180		
		E_{RED}	−220	t_{RED}	380		
		E_{ACT}	+750	t_{ACT}	100		

Significant developments were also accomplished by pulsed amperometry of amines, amino acids and organosulfur species using multistep potential waveform [20–25], and many applications in PED have appeared in the current literature, illustrating the maturity of the technique. A series of related detection waveforms are often grouped under the designation of integrated pulsed amperometric detection, IPAD [22,26–31]. These methods are similar to the three-step potentials waveform except that the potential during current sampling is not held constant but varied between a high and low value; being the oxidation of gold a reversible process, the gold oxide formed during the positive scan is reduced when returning to the lower potential value (see entry II in Table 1). As a result, the net signal is approximately the contribution coming from the analyte oxidation. As well described by LaCourse [6], IPAD is preferred over PAD for the determination of those analytes whose anodic detection is supposed to involve a catalytic action of surface electrode oxides. Definitely, the capability of detecting underivatized sugars, amino acids and amino compounds with high sensitivity is a major

advantage of pulsed amperometric detection over other detection methods. Thus, a novel six-step potential waveform (see entry III in Table 1) for the sensitive detection of amino sugars and amino acids following HPAEC at high pH was also proposed by Clarke et al. [32]. Generally, these multistep waveforms exhibit minimal background current due to electrode oxide formation and a good signal-to-noise ratio accompanied with a long-term reproducibility [9,32,33]. Indeed, several potential waveforms have been developed by manipulating potential values and time settings [34–36]. All these potential waveforms are so easy to implement that there may be a tendency to fall into the trap of assuming that all analytes are sensitively detected. Unfortunately, the electrochemical detection of amines and sulfur-containing compounds is complicated by adsorption phenomena on the electrode surface and by participation of surface oxides in the oxidation process. As the gold electrode is usually subject to a gradual loss of electroactivity, further improvements in the electrochemical selectivity and sensitivity of biologically significant compounds are required to extend the power and scope of pulsed amperometry. The great interest in the development of novel and well-performing waveforms in pulsed

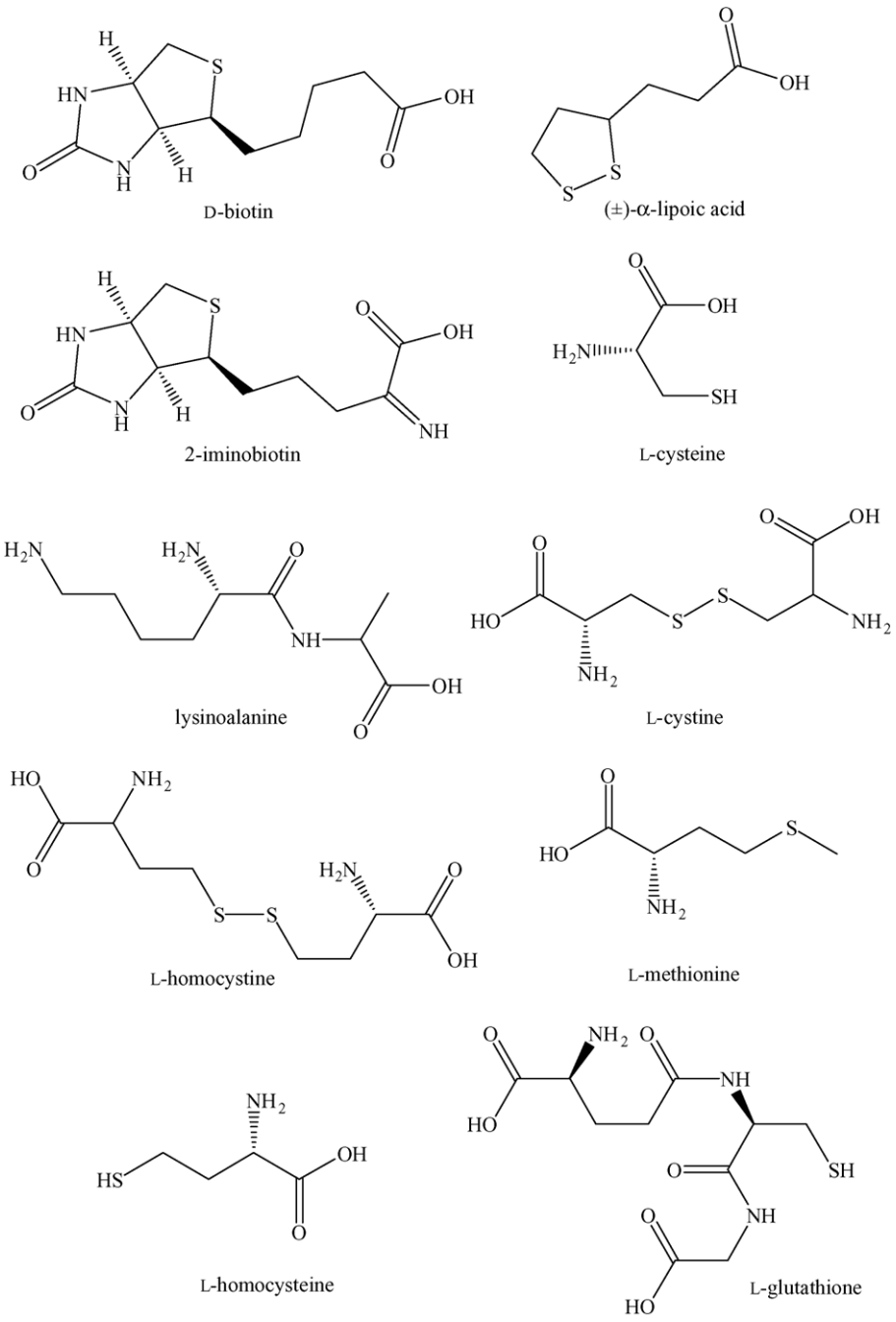


Fig. 1. Molecular structures of some N- and S-containing compounds of biological interest.

amperometry is also substantiated by the observation that many compounds which are now detected with great sensitivity, had been previously considered to be electroinactive on the basis of futile efforts of detection using a constant applied potential.

The present work describes a new approach to detect amino and thiocompounds, based on the electrochemical oxidation at a gold working electrode using an activated potential waveform (APAD), which is composed of three-potential steps plus a fourth and brief potential impulse at the end of the normal cycle which follows the detection potential (E_{DET}). During the application of this additional potential pulse, called activation potential (E_{ACT}), the formation of strong oxidizing electrochemical active species is suggested to occur at the gold electrode surface. A similar profile of potential waveform has already been shown very useful for As(III) detection at a Pt electrode in acidic media by Williams and Johnson in 1992 [37]. Here, the analytical performances in alkaline media of APAD at a gold working electrode in conjunction with HPAEC for the separation and detection of biologically significant analytes is presented. The structure of compounds investigated in this study is depicted in Fig. 1.

2. Materials and methods

2.1. Chemicals

D-Biotin (99%), DL-methionine (99%), L-cysteine (97%), L-cystine (99%), (±)-α-lipoic acid (99%), D-glucuronic acid sodium salt monohydrate (≥98%), lysinoalanine (95%), DL-homocysteine (95%), L-homocystine (98%), *N*-acetyl-L-cysteine (99%), L-glutathione reduced form (98%) were purchased from Sigma-Aldrich (Steinheim, Germany); 2-iminobiotin (≥98%) was from Fluka (Buchs, Switzerland). Sodium acetate (99%), sodium nitrate (99%), carbonate-free sodium hydroxide (50%, w/w) were purchased from Sigma-Aldrich (Steinheim, Germany). All the reagents used in this study were of the highest purity available and were used as received. Stock solutions were prepared with pure water supplied by Milli-Q RG unit from Millipore (Bedford, MA, USA). Sodium hydroxide solutions used as eluents were prepared by dilution of a carbonate-free 50% (w/w) NaOH solution in water, previously filtered with a 0.45 μm nylon membrane and degassed with nitrogen.

2.2. Chromatographic instrumentation

All chromatographic analysis were performed on a metal-free Dionex system (Dionex Corporation, Sunnyvale, CA) composed of a GP40 gradient pump, a Rheodyne injection valve (model RH9125, Cotati, CA, USA) with a 10 μL loop and a pulsed amperometric detector (model ED40). The flow-through detection cell is made from a 1.0-mm diameter gold working electrode and a standard combination pH-Ag|AgCl reference electrode using the half-cell Ag|AgCl as the reference electrode; the titanium body of the cell served as the counter electrode. All the separations were performed using Dionex microbore columns, CarboPac PA1 (2 mm × 250 mm) and AminoPac PA10 (2 mm × 250 mm), coupled with the corresponding guard columns. Separations were carried out at a flow rate of 0.25 mL/min or 0.15 mL/min by isocratic elution with NaOH (100–200 mM) with addition of $NaNO_3$ (10–50 mM) or NaOAc (10–400 mM). The plastic reservoir bottles (DX 5002 litres bottles, Dionex) were closed and pressurized with pure nitrogen to 0.8 MPa. The system was interfaced, via proprietary network chromatographic software (PeakNet^{TM}), to a personal computer, for instrumentation control, data acquisition and processing (Dionex).

2.3. Electrochemical measurements

Cyclic voltammetry (CV) studies were carried out using an Autolab PGSTAT 30 potentiostat from MetroOhm. A 2.0-mm diameter gold disk electrode was used as the working electrode (Radiometer CTV 101, Copenhagen). A saturated calomel (SCE) reference electrode and a platinum wire counter electrode were standard for CV analysis. RDE voltammetric experiments were performed using a model EDI 101 Rotating Disc Electrode analytical rotator (Radiometer, Copenhagen) and a CTV 101 Speed Control Unit. After dissolved O_2 removing by dispersion of N_2, all the experiments were carried out under a N_2 atmosphere at room temperature. Similar results were obtained without purging the electrochemical cell solution. The GPES software, version 4.8, in a potentiostat-interfaced PC was used for data acquisition. The electrochemical detection following chromatographic separations was accomplished by applying a pulsed waveform to the gold working electrode of the thin-layer electrochemical cell.

2.4. Standard and working solutions

Stock solutions of each compound were prepared in water at a concentration of approximately 10 mM and stored in a refrigerator (4 °C) not longer than 2 weeks. Working standard solutions were prepared on the day of use by suitable dilutions with water.

3. Results and discussion

3.1. Voltammetric responses of biotin at a gold-RDE

Voltammetric experiments at a gold rotating disk electrode (Au-RDE) were carried out to investigate the electrochemical responses of biotin, iminobiotin, lipoic acid, methionine and cysteine. It is well recognised that pulsed amperometric detection of carbohydrates and amines at Au electrodes is most favourable under alkaline conditions [38,39]. Hence, sodium hydroxide solutions were chosen for the voltammet-

ric investigations. The current–potential (*i–E*) curve of biotin in 0.10 M NaOH is shown in Fig. 2. The dashed line is the *i–E* curve recorded in the absence of analyte. During the positive scan starting at −0.8 V versus SCE, a broad anodic wave at $E > +0.20$ V was attributed to the surface oxide formation (AuOH and AuO) and the onset of oxygen evolution corresponded to the sharp current increase at potentials higher than +0.8 V. On the negative scan, the cathodic peak at about +0.05 V is due to the reduction of the surface oxide. In the presence of 0.5 mM biotin (solid curve), an increased anodic current was observed for its oxidation simultaneously with the surface oxide formation (positive scan). The large cathodic current corresponded to the reduction of gold oxides. It was found that the anodic wave between +0.1 and +0.7 V, namely the potential region where the anodic formation of surface gold oxide occurs [20], appeared unaffected by biotin concentrations at values greater than 0.5 mM. The anodic peak height of biotin was found to be independent of RDE rotation rate (100–5000 rpm) and linearly correlated to the potential scan rate (10–100 mV/s, not shown). It is also significant to note from the comparison of the cathodic peaks due to the oxide reduction that the presence of adsorbed biotin slightly suppresses the gold oxides formation during the positive scan. Most likely, such attenuation is caused by chemisorption of biotin at the oxide-free surface sites. These features are consistent with the suggestion that the major contribution to the anodic signal for biotin comes from the oxidative desorption of the analyte previously adsorbed at the oxide-free gold surface, at potential lower than 0.0 V.

Similar voltammetric response (i.e., *i–E* curves as in Fig. 2) was observed for the other compounds, such as lipoic acid, iminobiotin, methionine and cysteine (data not shown). These results confirm the occurrence of potential-dependent adsorption/desorption phenomena on the electrode surface, thereby, the preadsorption of organosulfur molecules on the oxide-free surface is required for their anodic detection. All these considerations are in good agreement with the electrochemical responses of amines and thiol compounds on gold

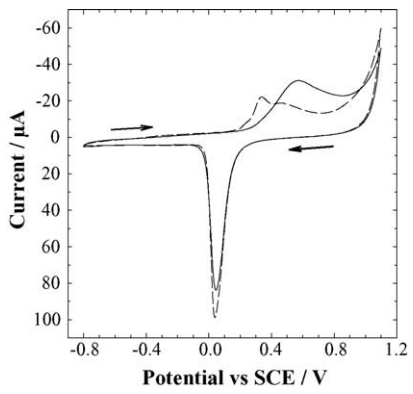


Fig. 2. Current–potential curves of biotin at a rotating disk gold electrode in 0.10 M NaOH. Solid line corresponds to the *i–E* curve obtained following addition of 0.5 mM biotin. Dashed line is the *i–E* background curve. Electrode rotation speed, 1000 rpm; scan rate, 100 mV/s.

electrodes well documented by Johnson and LaCourse [4]. It is believed that these compounds adsorb on the gold surface with the non-bonded electron pair of nitrogen and/or sulfur atoms. Under alkaline conditions, the adsorbed species are oxidatively desorbed simultaneously with the gold oxide formation process. As will be demonstrated in the next paragraphs, the intermediate products in the oxide formation (Au-OH) may act as active sites to catalyze the electrochemical oxidation of analytes at the gold electrode surface. Accordingly, a well-designed multistep potential waveform would be more suitable to detect sulphur-containing compounds following liquid chromatographic separations.

3.2. Separation conditions in HPAEC

Before dealing with the development and optimization of a new four-step potential waveform, data relevant the optimized chromatographic separation conditions are presented. Several papers describe separations of amino acids employing ternary gradient elution with water, 0.25 M NaOH and 1.0 M sodium acetate [8,32,40]. In an attempt to determine the mobile phase composition to be used in isocratic elution, preliminary experiments were carried out using a series of alkaline solutions, based on 100 mM NaOH with addition of sodium nitrate or acetate. By using the anion-exchange microbore column CarboPac PA1, we were able to perform an isocratic separation of biotin, iminobiotin, methionine, cysteine, cystine, lysinoalanine, glucuronic and lipoic acid by 100 mM NaOH + 15 mM $NaNO_3$ as an eluent at a flow rate of 0.25 mL/min.

3.3. Activated pulsed amperometric detection (APAD)

As illustrated above, the oxidation process of biotin and other organosulfur species at a gold working electrode takes place at potentials higher than +0.2 V versus Ag|AgCl. These potential values should be applied in order to assure a relatively high coverage of surface-active species. Unfortunately, for these potential values a large contribution to the oxidation signal is due to the background current resulting from the surface oxide formation. To minimize such an effect, several multistep potential waveforms have been proposed.

Polta and Johnson [41] reported the application of a potential waveform called reversed pulsed amperometric detection (RPAD), which exhibits a significant decrease in the background signal for several organosulfur compounds at a gold electrode. The RPAD differs from a conventional three-step potential waveform in that the order of application of E_{OX} and E_{RED} is reversed. A decrease in background current is expected because the Au oxide formation is immediately stopped with application of a negative potential step preceding the detection pulse. The E_{OX} step is responsible for both activation and oxidative cleaning of the electrode surface. However, this waveform provides an insufficient oxidative cleaning of the electrode surface with consequent distortion of the detection peaks. To eliminate such a disadvantage,

Johnson co-workers [37,42] proposed a pulsed amperometric waveform in which a brief activation pulse (E_{ACT}) was applied prior to the detection step. Such a waveform combines the advantages of both PAD and RPAD by allowing independent selection of the E_{ACT} and the oxidative cleaning (E_{OX}), which are managed separately. Such an activated waveform was employed at a platinum electrode in acidic conditions for the determination of As(III) [37] and cysteine [42]. The same idea of an activated potential waveform was applied here at a gold working electrode for the determination of thiocompounds in strongly alkaline solutions. In Fig. 3, is shown the activated waveform, elaborated modifying the traditional three-step potential profile upon the addition of a forth pulse for electrode activation, paying attention to keep the overall cycle time short enough to maintain peak integrity. The positive potential pulse (E_{ACT}) initiates the formation of the catalytically active $OH^{\bullet}$ species on the gold electrode surface and then, by stepping back to a less positive detection potential (E_{DET}), the rapid oxide growth is interrupted so that the conversion of AuOH to the inert AuO is halted. As described in the next paragraph, such a potential waveform exhibits a significant reduction of the background current in pulsed amperometric detection of nitrogen and sulfur-containing compounds whose oxidation mechanism is believed catalyzed by AuOH, but not by AuO [43]. Of equal importance are the oxidative cleaning and the reductive restoration of the oxide-free surface by the sequential application of $E_{OX} \gg E_{DET}$ and $E_{RED} < E_{DET}$. The power of this four-step potential waveform will be demonstrated by a comparative study in which different pulsed potential waveforms were applied to detect several significant analytes, following their separation by HPAEC under the optimized experimental conditions. Details are illustrated below.

3.4. APAD waveform optimization

Within a pulsed waveform, duration and potential values of each step may significantly affect the sensitivity and reproducibility of response. Thus, to further increase the detection capability of APAD waveform, a systematic investigation was carried out to optimize the values of E_{ACT}, t_{ACT} and E_{DET}, while all the other parameters were held constant. Waveform optimization was performed by injecting a standard solution, containing biotin, iminobiotin, methionine, cysteine, cystine, lipoic and glucuronic acid and plotting the amperometric signal-to-noise ratio (the average of three replicates) as a function of the parameter to be optimized. For each set of applied potential, the background noise was evaluated as the peak-to-peak value. In Fig. 4, is reported the signal-to-noise ratio as a function of E_{ACT}; this parameter was varied from +100 to +600 mV in 100 mV steps and then from +600 to +1000 mV by smaller steps of 50 mV. At potential values lower than +500 mV, very little of the electrode surface is activated and no sufficient amounts of catalytic species are most likely formed. At E_{ACT} > +600 mV, a large amperometric response was observed for all analytes up to +800 mV. At higher potential values, a stable and inert gold-oxide film, presumably composed of AuO and/or Au_2O_3, will form on the electrode surface that would inhibit the analyte adsorption, which in turn does not sustain the oxidation process. A potential value of $E_{ACT} \cong +750$ mV versus Ag|AgCl gave the best compromise between amperometric signal intensity and background noise. Although the E_{ACT} value is greater than the oxidation potential E_{OX}, its application is restricted to a very brief time period, thus avoiding the complete electrode surface oxidation. These considerations were found to be valid for all analyzed compounds with the exception of glucuronic acid, whose amperometric signal was found almost independent of the activation potential (Fig. 4). Such an effect was also confirmed for carbohydrates (i.e., glucose, fructose and

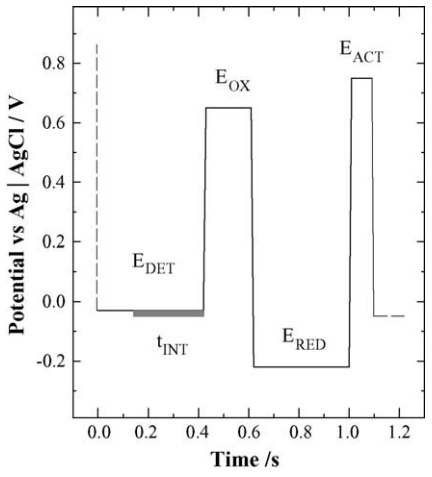


Fig. 3. APAD waveform for surface-oxide catalyzed anodic detection.

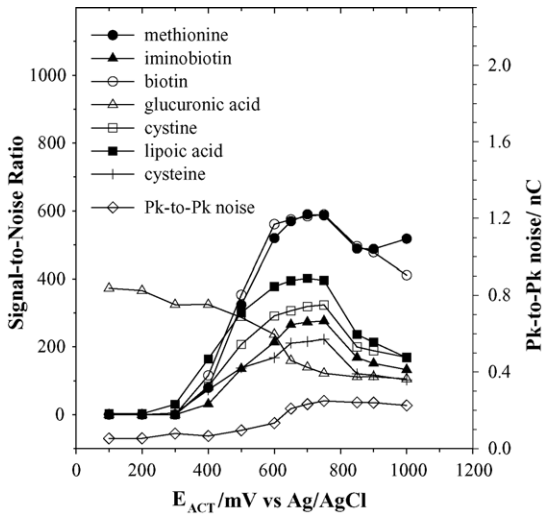


Fig. 4. Activation potential (E_{ACT}) effect of the four-step potential waveform on the signal-to-noise ratio. Other potential–time parameters as reported in Table 1, entry IV. The ordinate data was computed by the ratio of the integrated signal (nC) and the peak-to-peak noise (nC) evaluated in chromatographic separations.

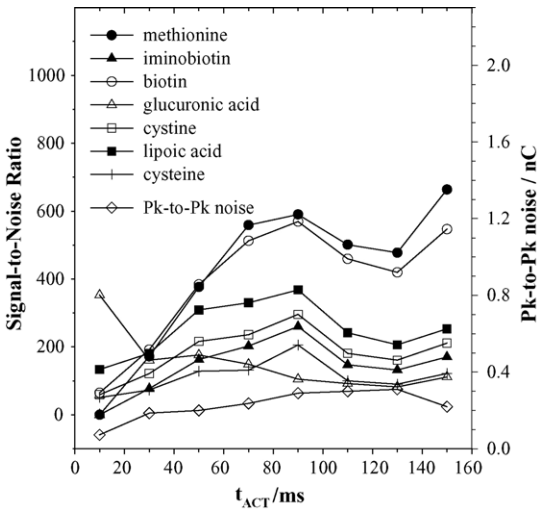


Fig. 5. Activation time (t_{ACT}) effect on the signal-to-noise ratio. Other experimental conditions as in Fig. 4.

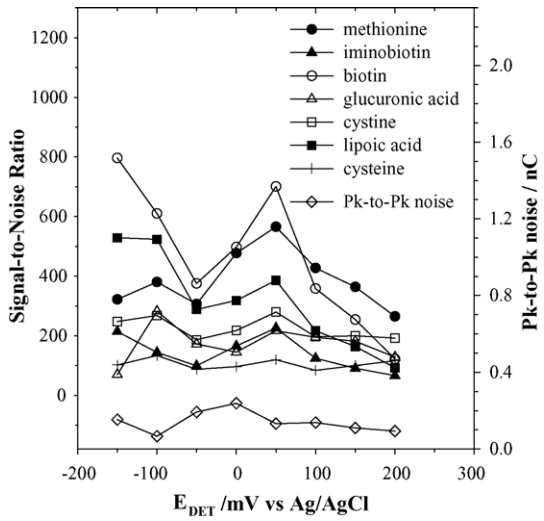


Fig. 6. Determination potential (E_{DET}) effect on the signal-to-noise ratio. Other experimental conditions as in Fig. 4.

galactose) and amino acids, such as proline, histidine and alanine.

The duration of the pulse activation, t_{ACT}, and the detection potential step, E_{DET}, also exerted a strong influence on the amperometric response; these parameters were optimized by a series of separate experiments. Fig. 5 illustrates the significant increase observed for methionine and biotin when the activation time was in the range 50–110 ms. As expected, when the electrode was activated at +750 mV for more than 100 ms, the resulting oxidation current of the analytes began to decrease. These findings suggests an optimum time value of t_{ACT} equal to 90 ms. An additional benefit of the APAD waveform is in the waveform cycle, which does not undergo a significant extension upon including a brief activation pulse ($t_{DET} + t_{OX} + t_{RED} + t_{ACT} = 1090$ ms). Finally, the detection potential (E_{DET}) optimization was carried out; its value was varied from −150 to +200 mV in 50 mV steps. As can be seen from Fig. 6, the optimum value of E_{DET} was found to be 50 mV versus Ag|AgCl. Hence, after the activation phase with an E_{DET} lower than E_{ACT} the adsorbed analyte coexists with the electro-catalytic $OH^{\bullet}$ species and an efficient anodic detection takes place. The optimized APAD waveform is described in Table 2. It provided the best signal-to-noise ratio for all compounds under investigation. This waveform was evaluated in terms of detection limit, linearity and reproducibility using biotin, lipoic acid, methionine, cystine and cysteine as model compounds (Table 3). Since preadsorption is a necessary step in the detection of organosulfur compounds, negative deviations from linearity for highly concentrated solutions were expected. Yet, the calibration graphs using peak areas were linear over two orders of magnitude with all correlation coefficients greater than 0.9992. The APAD waveform assures a linear response up to at least 20 μM, as observed for methionine, while the linear range

Table 3
Detection limits, linearity and reproducibility evaluated by HPAEC-optimized APAD[a]

	LOD		Linear range (μM)	r	% RSD[b]
	(μM)	(pg)			
Biotin	0.02	50	0.1–50	0.9997	1.9
Lipoic acid	0.03	50	0.1–70	0.9997	1.6
Methionine	0.02	30	0.1–20	0.9999	2.1
Cystine	0.08	200	0.1–70	0.9997	2.6
Cysteine	0.32	400	0.1–100	0.9992	2.2

[a] Chromatographic separation conditions as those reported in Fig. 7. Detection waveform APAD as described in Table 2.
[b] Intra-day relative standard deviations of the amperometric signals ($n = 7$).

Table 2
Optimized parameters of APAD waveform

Potential (mV vs. Ag|AgCl)			Time (ms)		
Parameter	General value	Optimized value	Parameter	General value	Optimized value
E_{DET}	−150 to +200	+50	t_{DET}	420	
			t_{DEL}	220	
			t_{INT}	200	
E_{OX}	+650		t_{OX}	180	
E_{RED}	−220		t_{RED}	380	
E_{ACT}	+100 to +1000	+750	t_{ACT}	10–150	90

increases up to 100 μM for cysteine. The best sensitivity was obtained for methionine with a LOD (S/N = 3) of 0.02 μM, corresponding to 30 pg for a 10 μL injection, which is an order of magnitude lower than that reported by using an IPAD waveform coupled to a microchromatographic separation [26]. Note that in the same reference [26] the LOD of cysteine was 0.2 pmol, which is rather lower than that found in this work, 3 pmol. In Table 3, are also reported the precision of peak areas observed upon injecting the same standard mixture under optimized experimental conditions. An acceptable precision was obtained by repeating the analysis seven times with an intra-day relative standard deviations lower than 2.6%.

Having available an optimized APAD waveform to detect organosulfur compounds, a comparison with other potential waveforms was made. Details of these potential waveforms are illustrated in Table 1. They are the classical three-step potential waveform (PAD), generally used to detect carbohydrates, alditols and uronic acids [15,44] and slightly modified under the present experimental conditions, the IPAD profile (waveform II) applied for the determination of amino acids in alkaline solutions [31], the six-step potential waveform (III) [32] for amino sugars and amino acids, and finally the optimized activated waveform. As can be seen in Fig. 7, neither the PAD waveform (plot A) nor the integrated waveforms II and III (plots B and C) offer the same high sensitivity obtained with APAD (plot D) for iminobiotin, biotin and lipoic acid. No positive signal was obtained for biotin and lipoic acid applying the integrated waveform II, and moreover, iminobiotin and biotin were not at all satisfactorily detected unless activated potential waveform was applied. By comparing the APAD response of biotin and lipoic acid with those obtained using the six-step potential integrated waveform III, a signal increase greater than 1200 and 200%, respectively, was observed. The ratios between the peak areas recorded using the two mentioned pulsed waveforms ($S_{APAD}/S_{Waveform\ III}$) evaluated for biotin and lipoic acid were 13.5 ± 0.6 and 3.2 ± 0.4, respectively. These results demonstrate the superiority of APAD over multistep potential waveforms for the determination of thiocompounds using anion-exchange chromatography. Moreover, as the peak area ratio values are characteristic of each compound, they can be used to provide additional proof of identity by employing a similar method to the use of UV wavelength ratios in combination with retention times [45]. The peak ratios are also useful to simplify the chromatogram by resolving closely co-eluting peaks of compounds exhibiting different electrochemical behavior [8]. For example, there is the possibility of selectively suppressing the biotin signal by applying the integrated waveform when a complex sample is analyzed in which it is difficult to investigate the chromatogram time window where the elution of biotin occurs.

In Table 4, data regarding the peak-to-peak noise, the root mean square noise level (rms) and LOD values of biotin (10 μL injected) are reported. The peak-to-peak noise was determined as average of five values corresponding to different baseline windows of each chromatogram; the detection limits were experimentally evaluated as three times the signal-to-noise ratios. Compared to other multistep waveforms, the APAD improves the S/N ratio by an order of

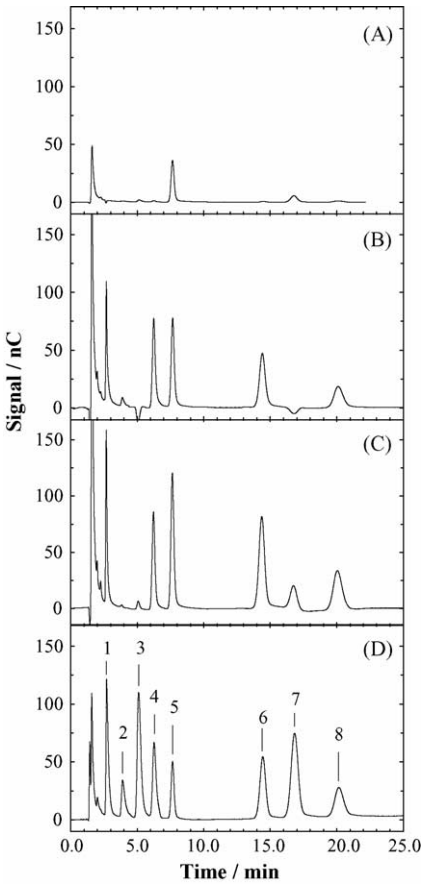


Fig. 7. Chromatographic separation of a mixed standard solution containing: (1) 25 μM methionine; (2) 5 μM iminobiotin; (3) 25 μM biotin; (4) 25 μM lysinoalanine; (5) 25 μM glucuronic acid; (6) 25 μM cystine; (7) 25 μM lipoic acid; (8) 25 μM cysteine. Eluent, 100 mM NaOH + 15 mM $NaNO_3$ at a flow rate of 0.25 mL/min. Column, microbore Dionex CarboPac PA1 plus guard column. Volume injected, 10 μL. Amperometric detection: (A) three-step PAD; (B) multistep waveform II; (C) six-step waveform III and (D) APAD as in detail indicated in Table 2.

Table 4
Peak-to-peak noise values and LODs evaluated for biotin by using the waveforms: three steps PAD (I) [15]; multistep waveform (II) [31]; six-step waveform (III) [32] and APAD (IV) with the optimized parameters[a]

Waveform	Peak-to-peak[b]	rms[c]	LOD	
			(μM)	(pg)
I	0.017 ± 0.009	0.005 ± 0.003	0.3	700
II	0.17 ± 0.02	0.038 ± 0.014	–[d]	–[d]
III	0.28 ± 0.05	0.050 ± 0.004	0.3	700
IV	0.34 ± 0.08	0.056 ± 0.016	0.02	50

[a] The chromatographic separation conditions as reported in Fig. 7.
[b] Values expressed in nC as an average of five measurements.
[c] rms, root mean square noise level.
[d] Presence of a negative peak.

magnitude and affords a stable baseline and a relatively low background noise. Notably, that among the investigated waveforms, APAD offers an outstanding sensitivity with a detection limit (S/N = 3) of biotin equal to 0.2 pmol on-column (50 pg), much lower than those obtained with the other potential waveforms. Similar considerations apply to iminobiotin and lipoic acid.

We also verified whether the activated potential waveform extends the scope of pulsed amperometry to a larger number of biologically relevant compounds, containing a sulfur moiety. Interestingly, the same positive behavior was observed for homocysteine, homocystine, *N*-acetylcysteine and glutathione. Their chromatographic separation was carried out by a microbore anion-exchange column, AminoPac PA10, eluted with 100 mM NaOH + 500 mM sodium acetate at a flow rate of 0.15 mL/min (data not shown). Both homocysteine and homocystine did not show significant difference in sensitivity upon applying the activated and the six-step waveforms as the peak areas APAD/Waveform$_{III}$ ratio ranged from 1.2 to 1.8. On the contrary, a significant $S_{APAD}/S_{Waveform\ III}$ ratio increment equal to 5.1 ± 0.6 and 11.6 ± 0.5 for glutathione and *N*-acetylcysteine, respectively, was observed. These results suggest that direct determination of thiocompounds, which are generally detected by applying the integrated pulsed waveforms, is greatly improved using the APAD potential–time setting at a gold working electrode. It is expected that understanding the relation between the oxidation process and hydroxide-reactive species may help to improve the application of this novel potential waveform. We have clear evidences that the activated waveform is also especially suitable for nucleosides and nucleotitides, so work is under way along this direction and will be reported on very soon.

4. Conclusions

Efforts to extend the electrochemical detection to amino- and sulfur-organic compounds have been challenging because of their adsorption on the electrode surface yielding an extensive fouling of noble metal electrodes. We have demonstrated the superiority of APAD over PAD and IPAD waveforms for the determination in alkaline solutions of thiocompounds characterized by the presence of disulfide and sulfide groups, while no marked improvement has been observed for amino acids and sugar-related compounds. With the possibility of catalyzing the anodic oxidation reactions of adsorbed analytes at gold electrodes, the activated four-step potential waveform seems to be very promising to sensitively detect biologically significant compounds. Apparently, the electrocatalytic species formed on the electrode surface at high pHs are those responsible to favor the oxidation process. While the concept of sulfur-containing compounds analysis is demonstrated, this new potential waveform seems to hold great interest for a broad spectrum of polar aliphatic analytes ranging from nucleosides, nucleotides and related compounds.

Acknowledgment

This work was supported by the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Rome). The authors are pleased to acknowledge financial support from the University of Basilicata. We are also pleased to acknowledge D. Montesano for his technical assistance and Dr. P. Villani for her contribution in performing some experimental work.

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