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Optimization of electrochemical detection in the high-performance liquid chromatography of lignin phenolics from lignocellulosic by-products

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SUMMARY

Free phenolic acids and aldehydes (*p*- and *o*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, vanillin, syringic acid, *p*-coumaric acid, syringaldehyde and ferulic acid) were detected in wheat straw extracts by high-performance liquid chromatography with a dual-cell electrochemical detector operated in the redox mode. Phenolics were oxidized with coulometric efficiency in the first cell (+1.00 V), then detected by reduction in the second cell (−0.20 V). Compared with the oxidative mode, the reductive detection mode has the advantage of being unaffected by large amounts of interferents eluting at the front of the chromatogram that interfere with the detection of small and early eluting compounds. Hydrodynamic voltammograms in the oxidative, reductive and screen-out modes are presented and the corresponding detection limits for real sample are discussed. Perfect linearity of response was found in the range $5 \cdot 10^{-7}$–$5 \cdot 10^{-5}$ *M* and detection limits were of the order of 50–500 fmol injected.

INTRODUCTION

High-performance liquid chromatography with electrochemical detection (HPLC–ED) has been successfully used for the determination of phenolic compounds in various matrices, such as water, vegetable materials and beverages[1–3]. Phenolic acids and aldehydes behave well in ED, being oxidizable at relatively low potentials. Under these circumstances, ED is often more sensitive and selective than the more common UV detection[4].

Recently, we applied HPLC–ED to the determination of phenolics in animal feeds made of lignocellulosic by-products[5], a large and inexpensive source of energy where the characterization of lignin-related phenolics is important owing to their antinutritional properties[6]. A standard method was used to extract different fractions

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of phenolics from lignocellulosics, *viz.*, oxidative hydrolysis with nitrobenzene for the determination of the total content of phenolics (or alkali-resistant lignin), dilute alkali solution for the so-called alkali-labile lignin and pH 7 buffer for the free phenolics. Owing to the selectivity of ED, analyses were carried out by direct injection of the extracts into the HPLC column, without the need for purification from UV-interfering substances (*e.g.*, nitrobenzene)[7]. However, the injection of free phenolic extracts often produced a large and tailing front peak that interfered with the early eluting compounds.

In an attempt to overcome this problem, a dual-cell electrochemical detector was operated in different modes, namely oxidative, reductive, screen-out and differential. This paper compares their application to both standard solutions and real samples, and proposes an experimental approach to the optimization of the detection of phenolics in lignocellulosics. Hydrodynamic voltammograms for several phenolic acids and aldehydes obtained in different modes are presented and other analytical parameters are discussed.

EXPERIMENTAL

HPLC system

A Model 590 pump (Waters Assoc., Milford, MA, U.S.A.) was connected in series to an Model LP 21 damper (Scientific Systems, State College, PA, U.S.A.), a Model 5020 guard cell (ESA, Bedford, MA, U.S.A.) set at +1.10 V, a Waters U6K injector, a Viospher C_6, 5 μm (Violet, Rome, Italy) reversed-phase column (150 × 4.6 mm I.D.) and an ESA 5011 dual-cell detector. The detector was controlled by an ESA Coulochem 5100 A module and chromatographic peaks were displayed on Leeds & Northrup Speedomax XL 681 (Leeds & Northrup Italiana, Milan, Italy) and Model 561 (Perkin-Elmer, Beaconsfield, U.K.) recorders.

The eluent was methanol–0.1% perchloric acid in water (12:88, v/v) at a flow-rate of 1 ml/min under a gentle stream of helium. HPLC-grade methanol (Carlo Erba, Milan, Italy) and laboratory-prepared deionized water were filtered through a 0.22-μm Millipore (Bedford, MA, U.S.A.) filtration unit under vacuum before use.

Hydrodynamic voltammograms (HDVs)

Oxidative mode (ox). Potentials from 0.00 to +1.00 V in increments of 0.05 V were applied to the first working electrode (D1) and the chromatograms of the standard solution (20 μl injected) were recorded at each potential. The current intensity of each peak was plotted against the applied potential, obtaining HDV_{ox} for the various compounds on D1. To obtain the HDV_{ox} on the second working electrode (D2), the first working electrode was fixed at +0.20 V and D2 was scanned from +0.20 to +1.00 V in 0.05 V steps, recording the resultant chromatograms.

Reductive mode (red). D1 was set at +1.00 V and D2 potentials were changed stepwise from +0.90 to −0.40 V in 0.10 V decrements and chromatograms of standard solution (20 μl injected) were recorded at each potential. Current intensity was plotted against the applied potential, obtaining HDV_{red} for the various compounds on D2.

Screen-out mode. D2 was set at +0.90 V and the D1 potential was scanned from +0.00 to +0.90 V in 0.10 V increments. The current intensity of each peak was

plotted against the applied potential, obtaining $HDV_{screen\text{-}out}$ for the various compounds.

Differential mode. The algebraic subtraction of the D2 output was obtained by direct connection of the output labelled CH1–CH2 on the rear of the control module to the recorder.

Standard solution

A working standard solution in the range $5 \cdot 10^{-7}$ *M* was prepared daily by 1:1000 dilution with water from a methanolic stock solution of the following compounds (Sigma, St. Louis, MO, U.S.A.): (1) *p*-hydroxyphenylacetic acid (final concentration $5.6 \cdot 10^{-7}$ *M*); (2) *o*-hydroxyphenylacetic acid ($5.4 \cdot 10^{-7}$ *M*); (3) *p*-hydroxybenzoic acid ($4.8 \cdot 10^{-7}$ *M*); (4) *p*-hydroxybenzaldehyde ($6.1 \cdot 10^{-7}$ *M*); (5) vanillic acid ($5.2 \cdot 10^{-7}$ *M*); (6) vanillin ($8.2 \cdot 10^{-7}$ *M*); (7) syringic acid ($3.6 \cdot 10^{-7}$ *M*); (8) *p*-coumaric acid ($4.4 \cdot 10^{-7}$ *M*); (9) syringaldehyde ($4.3 \cdot 10^{-7}$ *M*); (10) ferulic acid ($7.5 \cdot 10^{-7}$ *M*). The stock solution was stored in a refrigerator and kept for no longer than 1 week.

Calibration

The stock solution was diluted 1:10, 1:100, 1:200, 1:500 and 1:1000 with water to obtain single concentrations of phenolics ranging from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-7}$ *M*. Chromatograms were recorded with both D1 and D2 set at +1.00 V.

Lignocellulose analysis

Sample extraction. A 500-mg wheat straw sample (ground to pass a 0.2-mm sieve) was extracted with pH 7 buffer [containing EDTA disodium salt dihydrate (9.79 g/l), KH_2PO_4 (7.92 g/l) and $Na_2HPO_4 \cdot 2H_2O$ (7.22 g/l)] (20 ml) for 1 h under reflux[8]. After cooling, an aliquot of the supernatant was filtered through a 0.22-μm cartridge filter (Millipore) and injected into the HPLC system. Alternatively, the sample (5 g) was extracted with methanol (40 ml) at room temperature for 1 week with occasional shaking.

Chromatographic analysis. Chromatograms of untreated extracts were obtained in the oxidative mode (D1, +1.00 V), reductive mode (D1, +1.00 V; D2, −0.1 V), screen-out mode (D1, +0.30 V; D2, +1.00 V) and differential mode (D1, +1.00 V; D2, −0.10 V).

RESULTS AND DISCUSSION

The working potential range under the adopted chromatographic conditions was established by monitoring background currents of the flowing mobile phase at different potentials on both D1 and D2 (Fig. 1). Typically for this kind of detector[9], currents ranged from about +2 to about −2 μA for potentials from +1.00 to −0.30 V. Relatively constant currents (±1 μA) were observed from +0.80 to −0.20 V with sharper slope changes at the extreme potentials. These current values are relatively low and were not affected by the application or non-application of a guard-cell potential, thus indicating that the mobile phase was substantially free from electroactive impurities.

Hydrodynamic voltammograms in the oxidative and reductive modes (HDV_{ox}

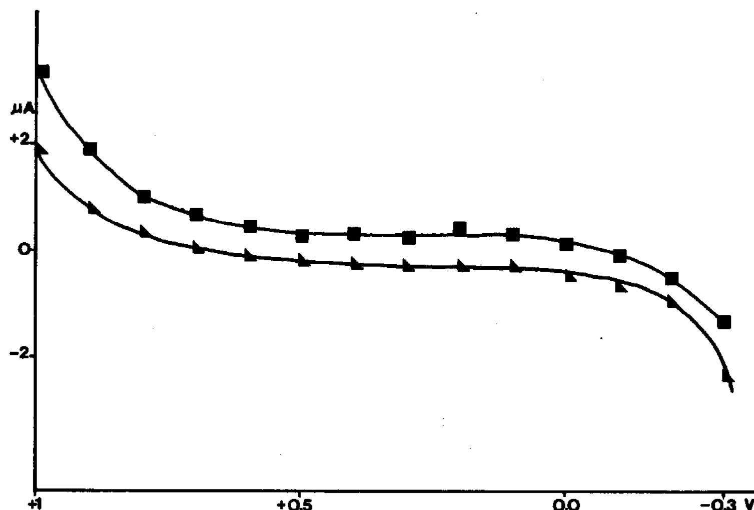

Fig. 1. Mobile phase background current *vs.* applied potential. Chromatographic conditions as under Experimental. ■ = D1; ▲ = D2.

and HDV_{red}, respectively) were recorded for several phenolic acids and aldehydes which are related to the three main monomeric constituents of lignin, namely the hydroxyphenyl moiety (*o*- and *p*-hydroxyphenylacetic acids, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde and *p*-coumaric acid), the coniferyl moiety (vanillic acid, vanillin and ferulic acid) and the sinapyl moiety (syringic acid and syringaldehyde). D1 and D2 showed similar HDV_{ox} curves for the various compounds. The half-wave potential ($E_{1/2}$) of the HDV_{ox} curves was in the range 0.40–0.50 V for the sinapylic, 0.50–0.65 V for the coniferylic and 0.65–0.80 V for the hydroxyphenylic compounds, thus indicating that the latter are less easily oxidizable than the former (Table I). Fig. 2 shows three typical examples of HDV_{ox} (upper curves). This experiment preluded to

TABLE I

HDV_{ox} AND HDV_{red} HALF-WAVE POTENTIALS ($E_{1/2}$) AND DETECTION LIMITS OF PHENOLIC ACIDS AND ALDEHYDES

Compound	*$E_{1/2}$ (V)*		*Detection limit (fmol injected)*
	HDV_{ox}	*HDV_{red}*	
Syringic acid	+0.40	+0.10	570
Syringaldehyde	+0.50	+0.10	400
Vanillic acid	+0.50	+0.10	65
Vanillin	+0.60	+0.10	75
Ferulic acid	+0.65	+0.10	82
p-Hydroxyphenylacetic acid	+0.65	−0.10	38
o-Hydroxyphenylacetic acid	+0.70	−0.10	40
p-Coumaric acid	+0.70	−0.10	44
p-Hydroxybenzoic acid	+0.80	−0.10	40
p-Hydroxybenzaldehyde	+0.80	0.00	490

the choice of the optimum potential for the oxidative mode detection and for subsequent reductive and screen-out mode operations. It was also useful for identification purposes via comparison of the HDV of the standards with those of the unknowns.

Typical HDV_{red} are reported in Fig. 2 (lower curves). It is interesting that the reductive currents are lower than the oxidative currents and that the hydroxyphenylic

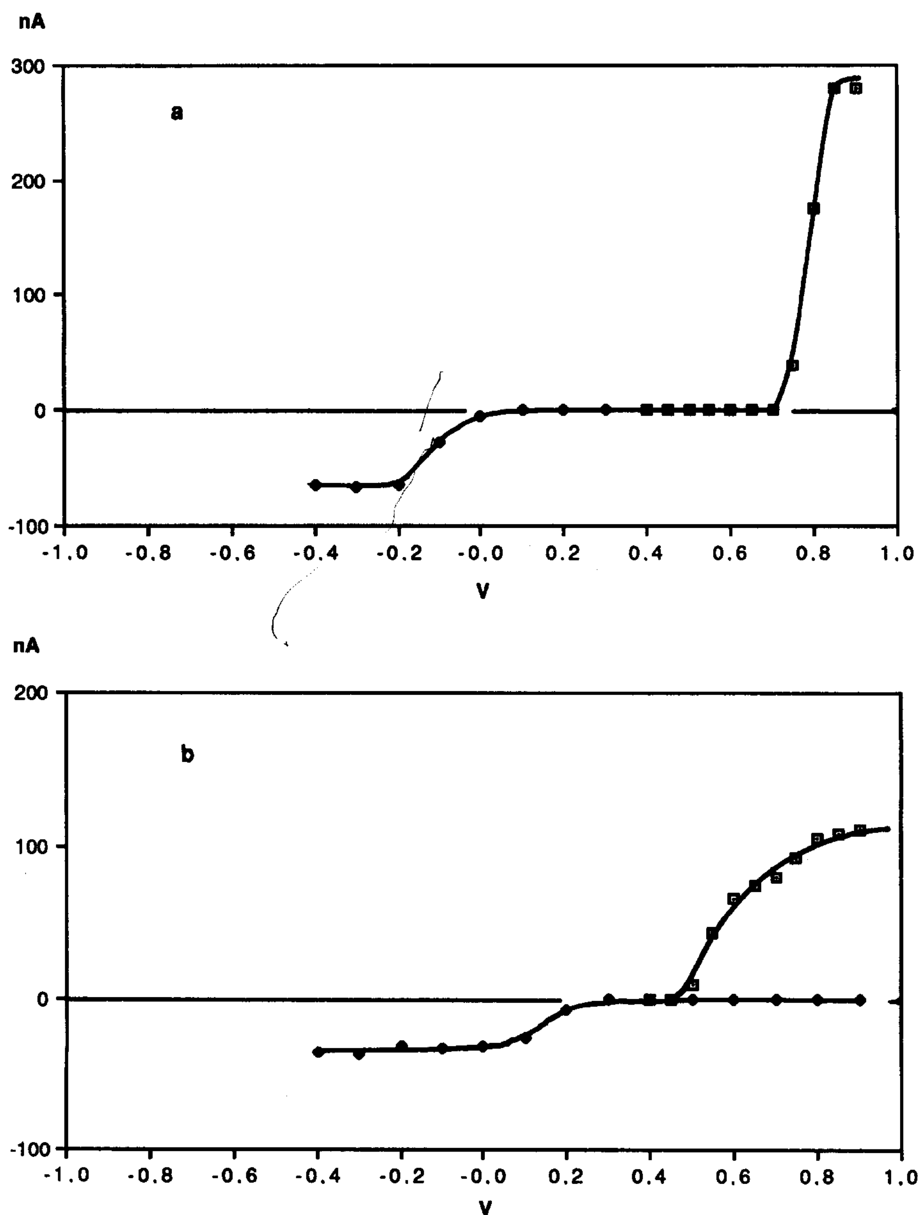

Fig. 2.

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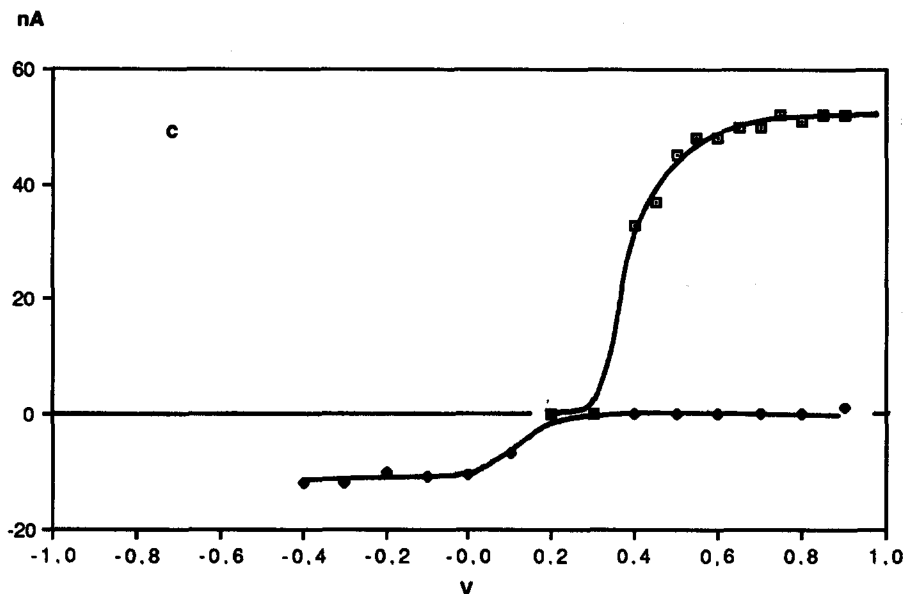

Fig. 2. Hydrodynamic voltammograms in oxidative and reductive detection modes (□ = HDV_{ox}, upper curves; and ◆ = HDV_{red}, lower curves) of representative phenolics: (a) *p*-hydroxybenzoic acid; (b) vanillin; (c) syringic acid. For chromatographic conditions see experimental section.

compounds, which are the more difficult to oxidize, are also the less easily reduced, with $E_{1/2} \approx -0.1$ V.

Detection limits are reported in Table I. These were of the order of 50–100 fmol injected (5–20 pg injected) for all compounds except *p*-hydroxybenzaldehyde, syringic acid and syringaldehyde [400–600 fmol injected (10–60 pg injected)] in reductive mode. These values are about two orders of magnitude lower than those obtainable by UV detection. The oxidative mode can also reach these detection limits, but the detection of the early-eluting peaks at low concentrations is often hindered by a large interfering front peak. In a detailed comparison of UV and electrochemical detection of phenolic compounds, Hayes *et al.*[4] obtained UV detection limits similar to our results, whereas the electrochemical detection limits were about one order of magnitude higher than those reported here, owing to the poorer sensitivity of the amperometric detector used in their work.

D1 can be used to screen out background currents and chromatographic interferents that are oxidized at potentials lower than that for the analyte. In the screen-out mode, detection is carried out at D2, and the D1 potential should be set at a value that is sufficiently high to oxidize impurities, but not so high as to reduce significantly the analyte response. As our compounds covered a fairly wide range of oxidation potentials ($0.4 < E_{1/2} < 0.8$), an optimum screen-out D1 potential was chosen on the basis of the $HDV_{screen\text{-}out}$ of each compound. Relevant examples are shown in Fig. 3. These HDV_{ox} curves were generated by plotting compound responses at D2 (+0.9 V) *vs.* D1 potentials. The maximum allowable D1 potentials for phenolic compounds to be detected at D2 without significant losses of response were +0.2 and +0.3 V for

syringic acid and syringaldehyde (sinapylic compounds), respectively, +0.3 V for coniferylic compounds and +0.4 V for *p*-hydroxyphenylic compounds (+0.6 V for *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde).

As a result of these preliminary experiments, it was concluded that, given a useful working potential interval ranging from −0.3 to +1.0 V, optimum detection conditions for the studied phenolic compounds were as follows: D1, +1.00 V (ox-

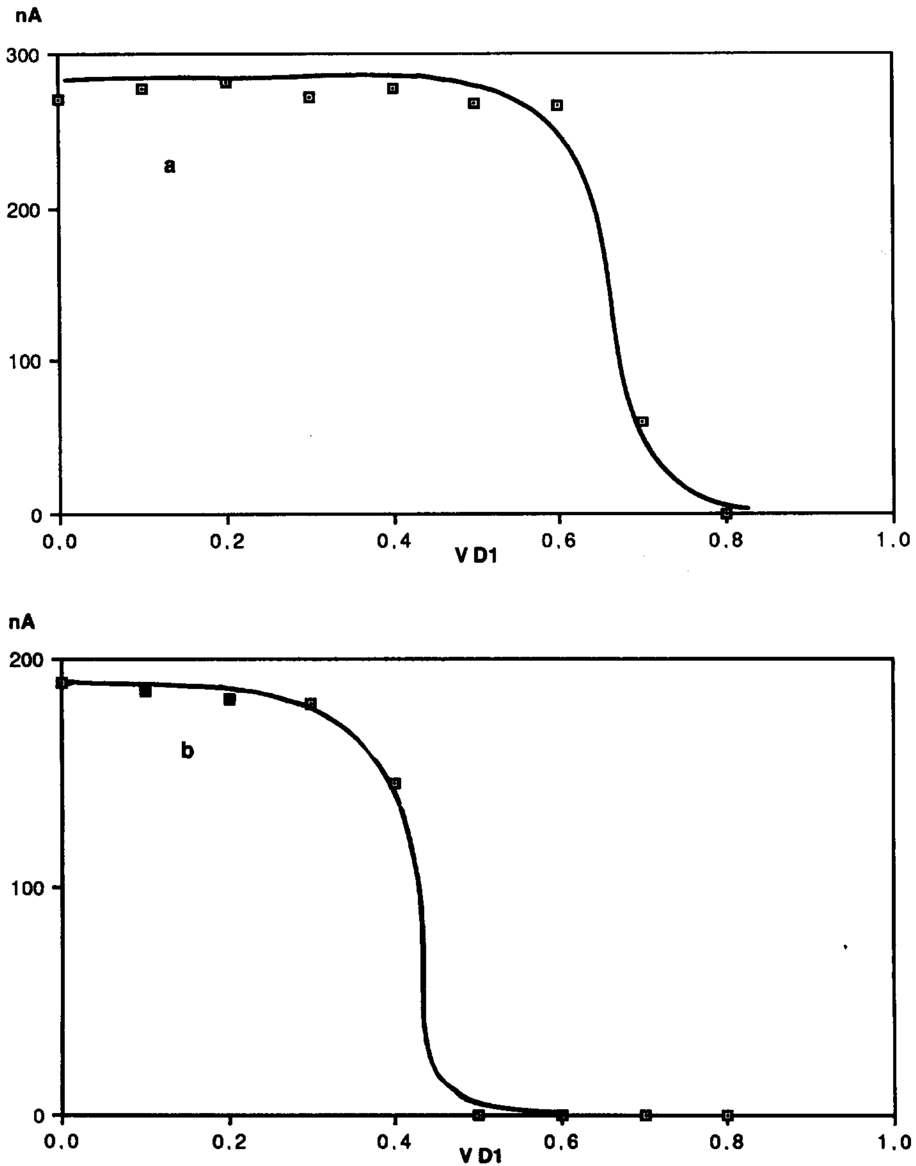

Fig. 3.

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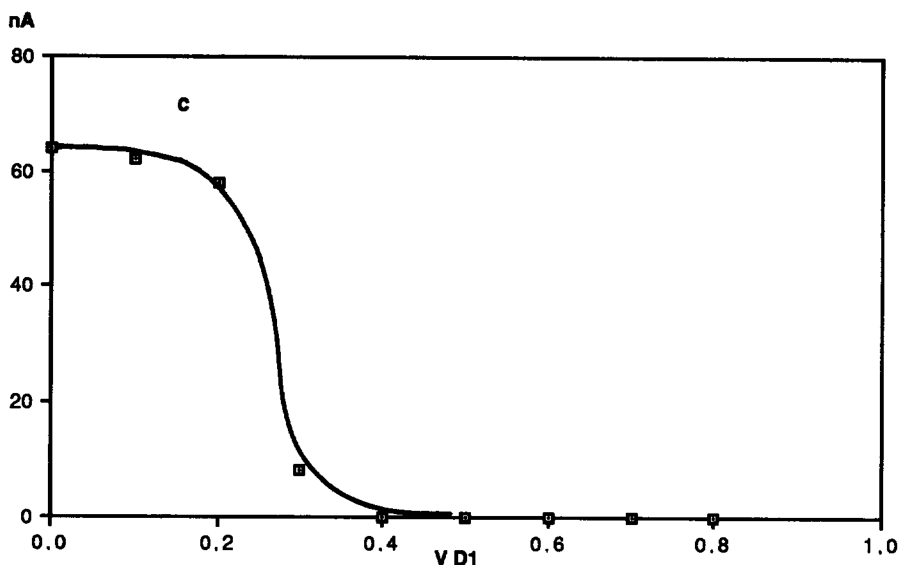

Fig. 3. HDV screen-out of (a) *p*-hydroxybenzoic acid, (b) vanillin and (c) syringic acid. Chromatographic conditions as under Experimental.

idative mode) (Fig. 4); D2, −0.20 V and D1, +1.00 V (reductive mode) (Fig. 4); D2, +1.00 V and D1, +0.3 V (screen-out mode).

The detection response was linear over the range of concentrations ($5 \cdot 10^{-7}$–$5 \cdot 10^{-5}$ *M*) used for the calibration (R = 1.00). The oxidative efficiency of D1 was more than 90%, regardless the concentration of the analyte.

The free phenolic extract from straw was firstly run in the oxidative mode. Because detection at + 1.00 V resulted in excessive current limits, the detection potential was lowered to +0.80 V, where all the compounds still have good responses (Fig. 5). A large and tailing front peak made detectable only major peaks with retention times longer than 7 min. This peak was present also in the chromatograms with UV detection. Any option taken into consideration to improve the chromatogram would mean either changing the extraction classical method (by changing the buffer composition or concentration or by using another solvent) or changing the detection mode. However, neutral buffers are reported to have better extraction efficiencies for phenolics from forages than other solvents[10] and, hence, can be considered as a standard extraction procedure. On the other hand, simple dilution of the extract was not possible as minor peaks would fall below the detection limit. Examination of the electrochemical behaviour of the front peak showed that its response was almost zero at +0.30 V, reaching a maximum at +0.80 V, thus ruling out the possibility of using the screen-out detection mode. Reductive mode detection resulted in an improved chromatogram (Fig. 5), with a fairly horizontal baseline and minor peaks detectable from retention times of *ca.* 3 min. Differential mode detection (subtracting the D2 reductive output from the D1 oxidative output) resulted in an increase in peak height ranging from 14 to 51% of the original oxidative peaks (Table II). The D2 gain was a compromise between increasing the peak height while minimizing the noise and base-

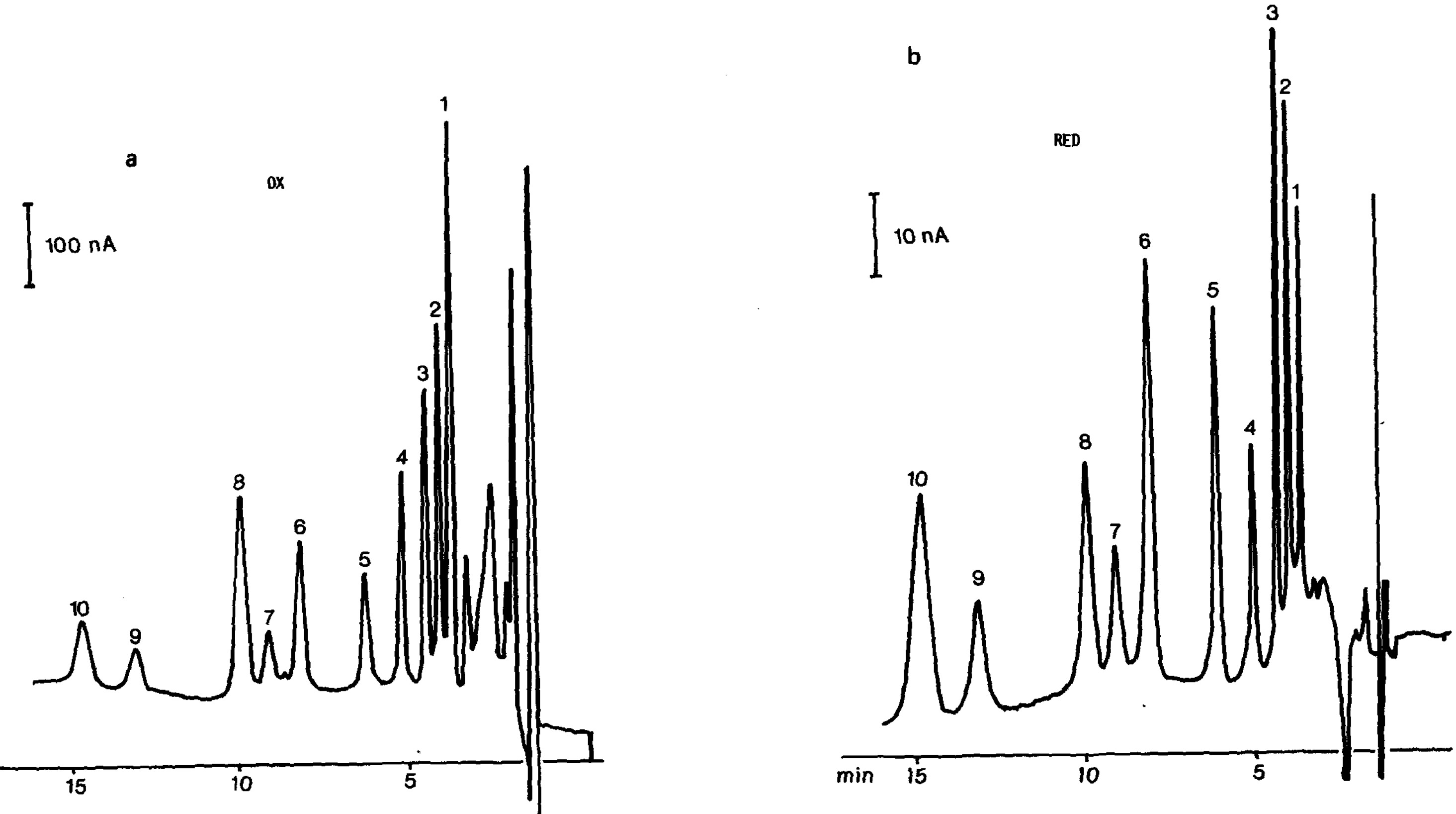

Fig. 4. Standard phenolics (*ca.* $5 \cdot 10^{-7}$ *M*) detected in (a) oxidative (first working electrode, D1 = + 1.00 V) and (b) reductive mode (second working electrode, D2 = − 0.20 V, D1 = + 1.00 V). Chromatographic conditions and peak numbering as under Experimental.

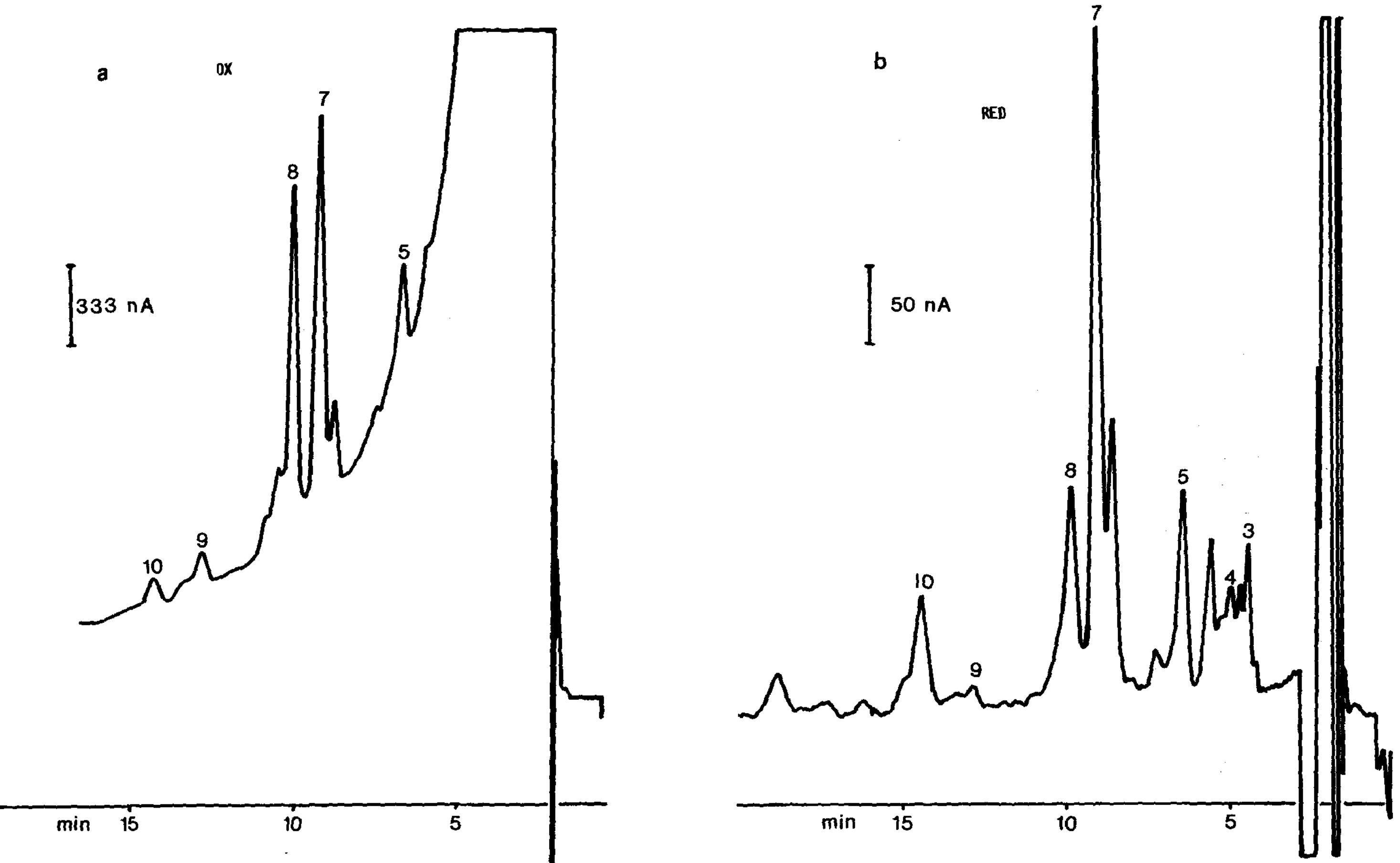

Fig. 5. Wheat straw buffer extract. (a) Oxidative (D1 = +0.80 V) and (b) reductive (D2 = −0.20 V, D1 = +1.00 V) mode detection.

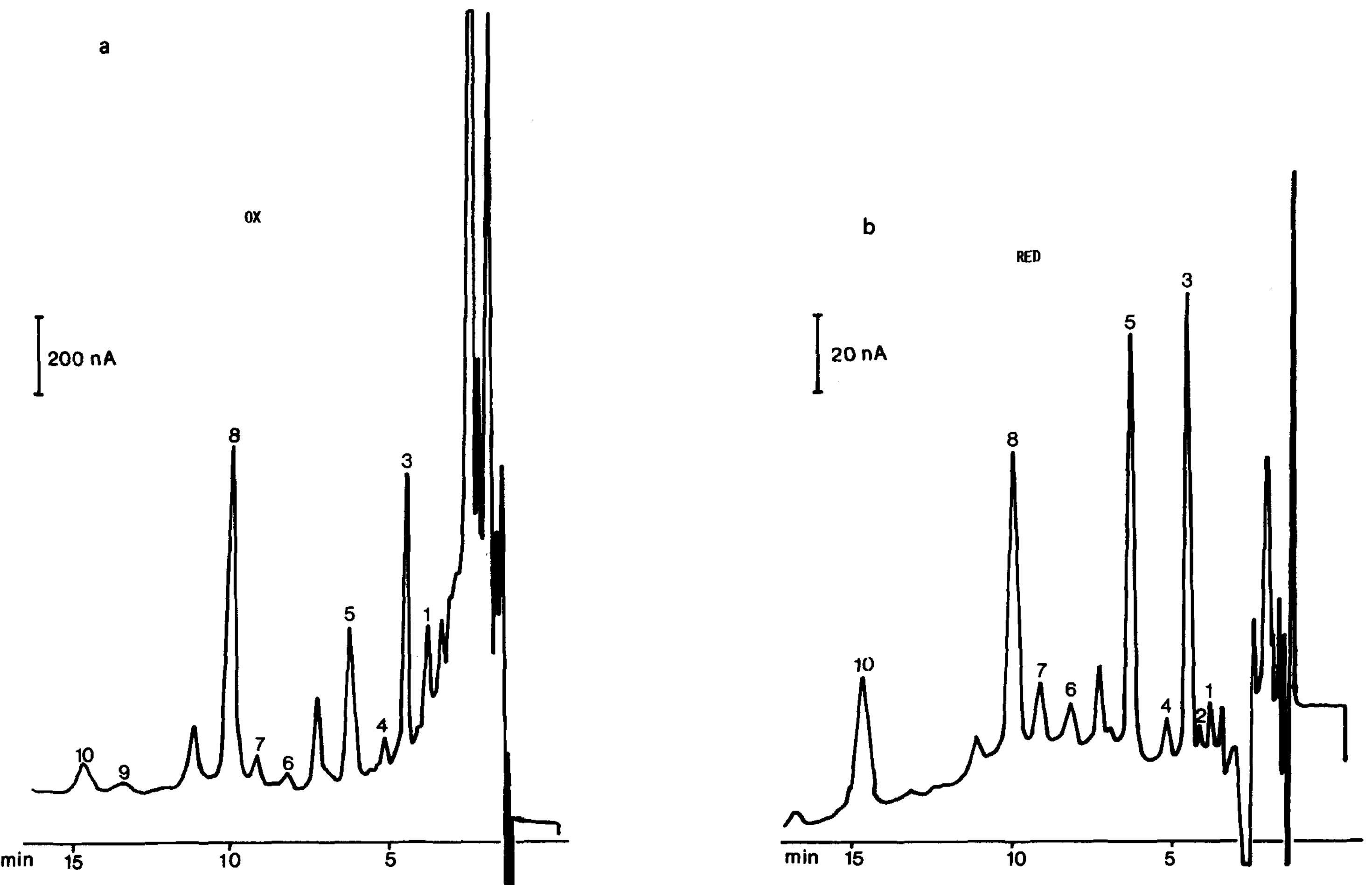

Fig. 6. Wheat straw methanol extract. (a) Oxidative (D1 = +0.80 V) and (b) reductive (D2 = −0.20 V, D1 = +1.00 V) mode detection.

TABLE II

PEAK HEIGHTS (mm) OF STANDARD PHENOLICS ($5 \cdot 10^{-8}$ *M*) DETECTED IN OXIDATIVE (D1, +1.00 V, GAIN ×10 × 50), REDUCTIVE (D2 −0.20 V, GAIN ×100 × 10) AND DIFFERENTIAL MODES (D1 − D2) WITH % INCREASE IN D1 − D2 WITH RESPECT TO D1

Compound	*D1*	*D2*	*D1 − D2*	*Increase (%)*
p-Hydroxyphenylacetic acid	85	10	98	15
o-Hydroxyphenylacetic acid	55	16	75	36
p-Hydroxybenzoic acid	70	28	102	46
p-Hydroxybenzaldehyde	32	9	40	25
Vanillic acid	37	17	56	51
Vanillin	25	14	38	52
Syringic acid	11	5	16	45
p-Coumaric acid	37	8	42	14
Syringaldehyde	9	4	13	44
Ferulic acid	12	7	18	50

line drift. In an attempt to reduce the front peak, a differential mode was operated by setting D1 at +0.80 V (where both the front peak and the phenolics are detectable) and D2 also at +0.80 V (where the front peak only is visible, because it is too large to be coulometrically oxidized at D1, whereas the phenolics are not detectable). The result was unsuccessful, because it was impossible to obtain algebraically zero values from the subtraction of a peak exceeding the current limits.

The other approach to improvement, that is, changing the extracting solvent, was also tested, replacing pH 7 buffer with methanol and diluting the extract with water (1:10) before injection. The front peak was smaller than with buffer extraction, but smaller peaks were detectable better in the reductive mode than in the oxidative mode (Fig. 6). Further, the methanolic extract yielded a different chromatographic profile than the buffer extract, which would make a comparison with the standard extraction procedure impossible.

To conclude, the reductive mode detection of free phenolics in lignocellulose offers the advantages of much better sensitivity than UV detection and of a chromatogram unaffected by the large and tailing front peak present in the oxidative mode, allowing the injection of extracts with no need for purification steps.

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