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Note

Electrochemical detection in the high-performance liquid chromatography of polyphenols (vegetable tannins)

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Polyphenols (vegetable tannins*)[1] constitute one of the most distinctive groups of secondary metabolites in higher plants[2]. Their uniqueness lies not only in their molecular size and polyphenolic character but also in their ability to complex strongly with proteins[3], carbohydrates, nucleic acids, alkaloids and minerals.

Studies of this widely distributed class of natural compounds are not only of scientific interest but are also of considerable practical significance. The characteristics of many plant products, *i.e.*, taste, palatability, nutritional value, pharmacological and toxic effects and microbial decomposition, substantially depend on the polyphenols they contain. Haslam[4] subdivided these polyphenols into two groups, hydrolyzable tannins (esters of glucose with hydroxyphenolic acids) and condensed tannins [polymers of flavonoid precursors such as flavan-3-ol (catechin)].

Despite the advent of impressive new methods of separation[5–7] and structure determination[8], the investigation of plant polyphenols is not yet free of problems. High-performance liquid chromatography (HPLC) has been successfully applied to the separation of condensed tannins[5,9], but the enormous inherent complexity of many plant extracts necessitates time-consuming pre-purification of the crude extracts prior to HPLC. Even so, problematic separations occur and interferences caused by matrix elements are more important than the detector noise. Chromatograms sometimes show unpredictable overlapping peaks because the interference patterns are different from one plant to another. Doubts might therefore arise about the uniformity of the UV-monitored peaks.

Electrochemical detection (ED) has become widespread in phenolic trace analysis[10,13] and its application to the analysis of vegetable tannins merits attention. The glassy carbon detector possesses greater selectivity than the UV detector because of the smaller number of electroactive substances as compared to UV-absorbing compounds. Problems related to the theory of ED have been discussed elsewhere[14,15]. In particular, Nagels and Creten[16] evaluated ED for of four phenolic acids in ethanolic extracts from plant leaves.

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* Hereinafter the terms tannins and polyphenols will be used as synonymous.

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This paper reports on the ED of some procyanidin oligomers so as to improve their HPLC analysis by an enhanced selectivity and sensitivity.

EXPERIMENTAL

Apparatus

HPLC determinations were carried out with a Waters liquid chromatograph, equipped with a Rheodyne Model 7010 sample injector (20-μl loop), a Model M-45 pump and a Model 440 UV detector. A reversed-phase column (120 mm × 4.6 mm) Viospher C6 5 μm, was employed for all separations. The eluent was methanol–0.1% perchloric acid in water (15:85, v/v). The electrochemical detector was a Metrohm Model 656 equipped with a three-electrode detection cell (Model EA 1096/2) with an internal volume of *ca.* 1.3 μl. A Metrohm VA 641 potentiostat was used and the detector output was displayed on a Perkin-Elmer Model 561 recorder. The glassy carbon working electrode was polished daily using alumina powder (0.3 μm). UV absorbances were measured at 280 nm and displayed on a Leeds and Northrup Speedomax recorder. The UV and electrochemical detectors were connected in series in that order.

Reagents and natural extracts

(+)-Catechin (Sigma) was used as a monomer standard. Procyanidin oligomers, dimer B-2, trimer C-1 and tetramer (Fig. 1), were kindly supplied by A. G. H. Lea, Cadbury Schweppes, Lord Zuckerman Research Centre, Whiteknights, Reading

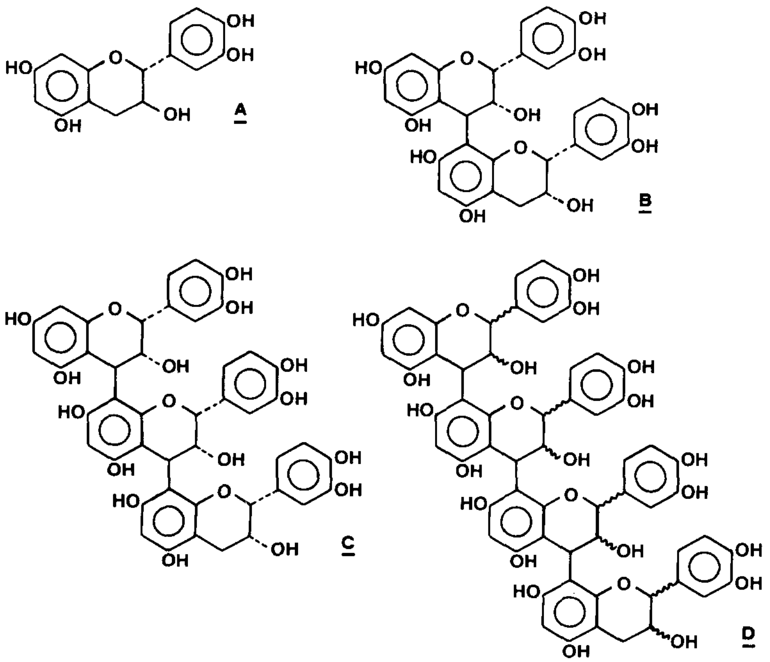

Fig. 1. Polyphenols: (A) (+)-catechin; (B) dimer B-2; (C) trimer C-1; (D) tetramer.

(U.K.). They were isolated by adsorptive chromatography on Sephadex LH-20 (eluent ethanol) of tannin-rich extracts from bittersweet cider apples, as described[17]. The nature of the compounds was confirmed by chemical degradation, NMR and fast atom bombardment mass spectrometry (FAB–MS)[8,9].

Free phenols in maize silage

The phenols in a sample of maize silage (3.00 g) were extracted with Neutral Detergent Solvent (NDS)[18] (150 ml) for 30 min with heating under reflux. After addition of 1% α-amylase aqueous solution, an aliquot of the filtrate (5 ml) was acidified and extracted with dichloromethane (3 × 30 ml). After drying over anhydrous sodium sulphate, the organic solvent was evaporated and the residue was dissolved in water (2 ml) and injected for HPLC.

RESULTS AND DISCUSSION

The capacity factors, k', of the compounds studied are collected in Table I; no further attempts (pH or eluent changes) were made to optimize the separation conditions because our interests focused on testing the analytical possibilities of ED.

It has been shown previously that polyphenols can be separated successfully by gradient elution[5]. Nevertheless, isocratic conditions were used for the present work because a baseline drift occurs when gradient elutions are employed with ED.

Fig. 2 shows the chromatograms of the individual compounds. Catechin and dimer B-2 were practically pure, whereas trimeric and tetrameric procyanidins contained substances with similar capacity factors to that of the dimer. The quantity of these impurities is about 40% of the respective fraction. Their nature has been discussed by Self *et al.*[8], but some additional considerations will be described below when their electrochemical behaviour is examined.

The HPLC separation of a mixture of the four polyphenols simultaneously detected by UV and ED is shown in Fig. 3. Catechin showed higher responses than the polymers, particularly in ED. This is consistent with an easier oxidation of the monomer with respect to the more complex polymeric molecules on the glassy carbon surface.

Polyphenol calibration curves for UV and ED (Fig. 4) showed an overall higher response of ED, with the above-mentioned marked selectivity towards catechin. The responses of the four compounds followed linear regression equations in the range of 0.010–0.200 nmol injected, correlation coefficients being 0.999. The detection limits, based on the quantity necessary to give a response twice the noise level, were

TABLE I

HPLC CAPACITY FACTORS, k', OF CATECHIN AND PROCYANIDIN OLIGOMERS

Compound	k'
Catechin	2.27
Dimer B-2	3.09
Trimer C-1	6.45
Tetramer	8.00

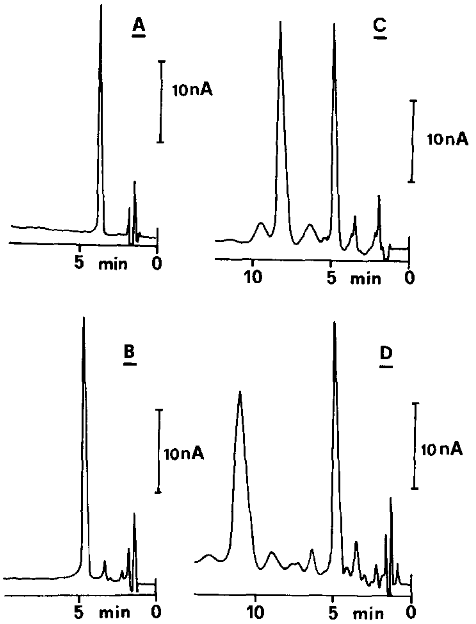

Fig. 2. HPLC separation of the individual polyphenols detected by ED: (A) (+)-catechin; (B) dimer B-2; (C) trimer C-1; (D) tetramer. For chromatographic conditions see Experimental section.

about 0.01 nmol at 1.1 V (for ED) and 0.01 nmol at 0.005 a.u.f.s. (for UV), with the best value for catechin being 0.005 nmol.

Fig. 5 illustrates the electrochemical behaviour of catechin and procyanidin oligomers, dimer B-2, trimer C-1 and tetramer, as a function of the applied potential.

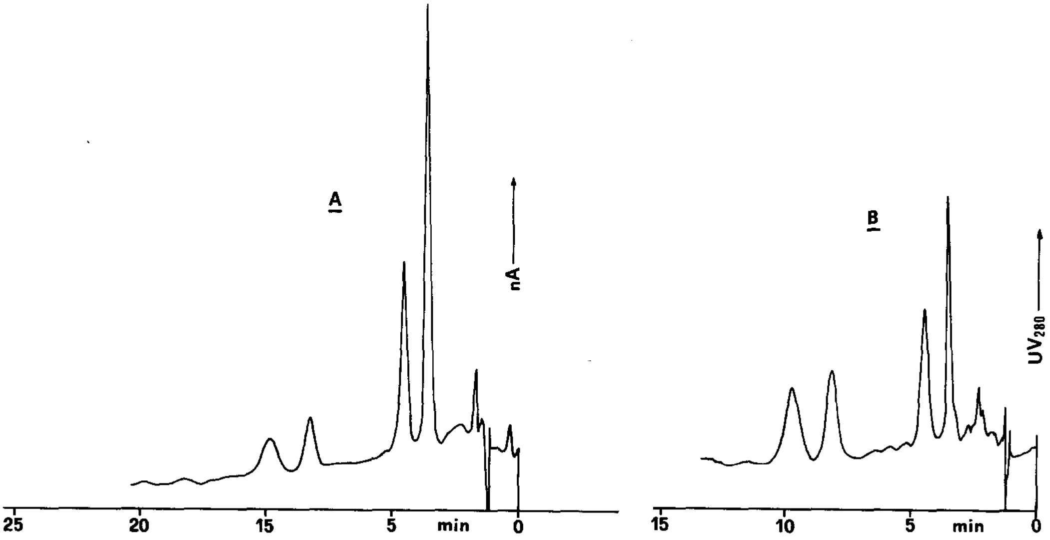

Fig. 3. HPLC separation of the polyphenol mixture: (A) ED (+1.10 V); (B) UV detection (280 nm). Chromatographic conditions as in Experimental section.

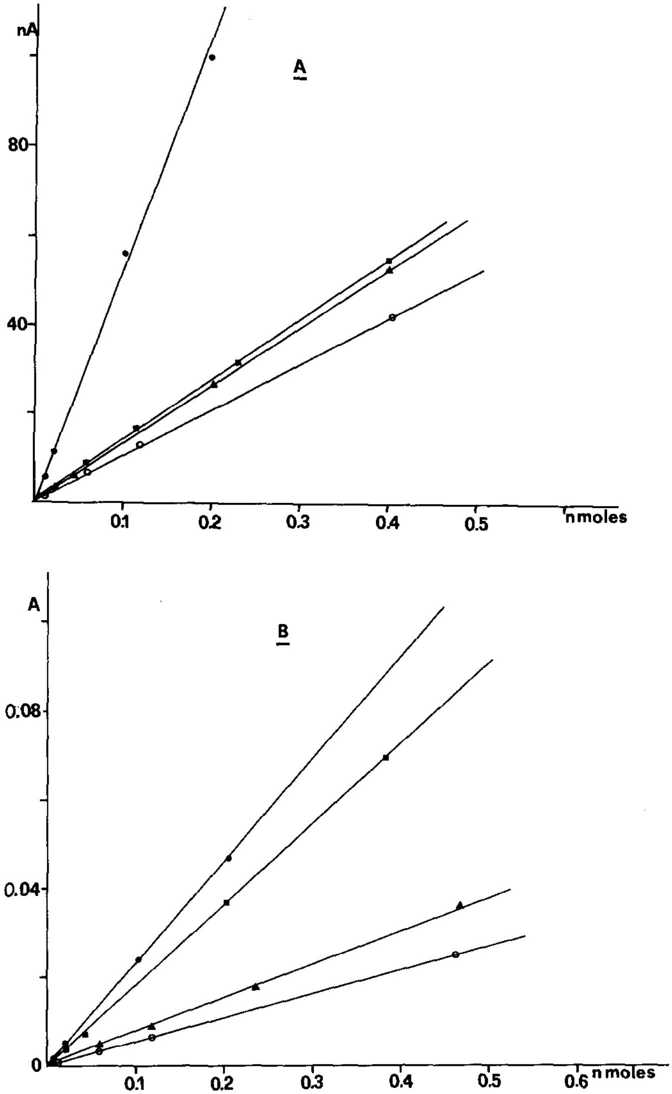

Fig. 4. Calibration graphs of polyphenols: ●—●, (+)-catechin; ■—■, dimer B-2; ▲—▲, trimer C-1; ○—○, tetramer. (A) ED (+1.10 V); (B) UV (280 nm).

The responses in terms of peak heights depend on the electrode conditions and can vary somewhat from one experiment to another, but this does not invalidate the oxidation profiles and the electroactivity range of the substances. The catechin oxidation curve clearly showed a plateau, approximately between +0.85 and +0.65 V. A possible explanation of this is the different oxidation potential of the *ortho-* and *meta-*dihydroxy-substituted phenyl moieties of catechin, as has been observed for simple phenols[16]. The plateau was less evident for the dimer B-2; the trimer and tetramer profiles showed only a change in slope. The oxidation curve for the compound present as an impurity in the trimeric fraction is different from that of the dimer B-2, although their capacity factors are coincident. This suggests that the two compounds may be different isomers. On the other hand, although the oxidation curve for the impurity present in the tetrameric fraction is similar to that for the trimer C-1, their capacity factors are different. This is consistent with the tentative identification of this impurity as a trimer (but not C-1), as reported elsewhere[8].

As an example of the possible application of ED to the HPLC analysis of natural mixtures, the chromatograms for an extract of the free phenols in maize

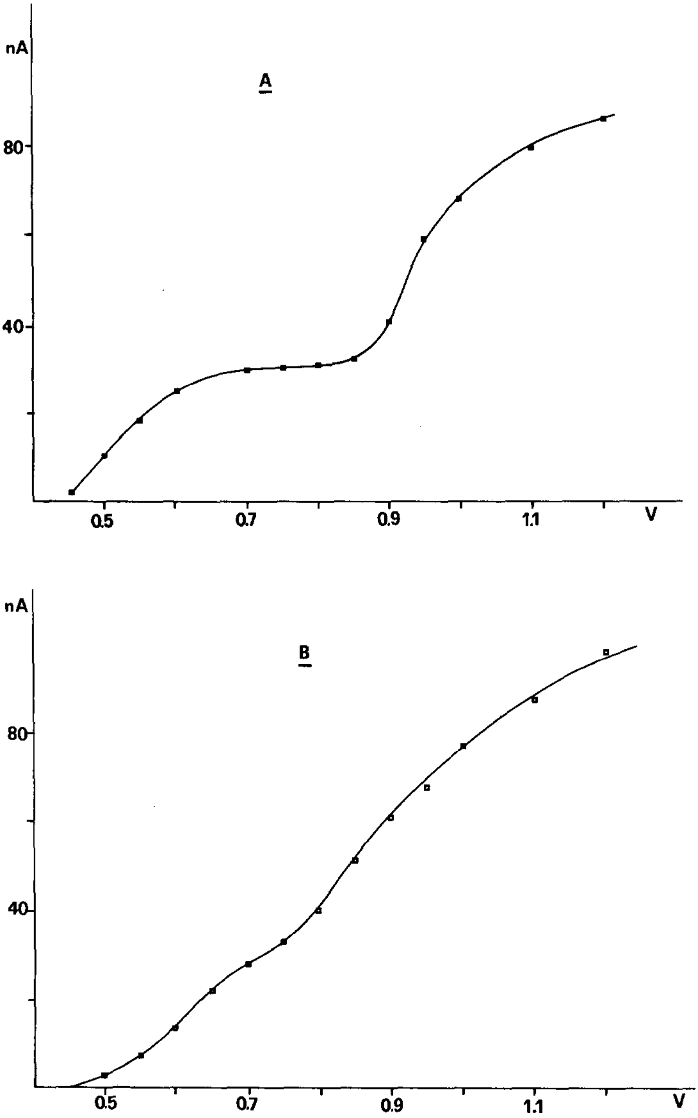

Fig. 5.

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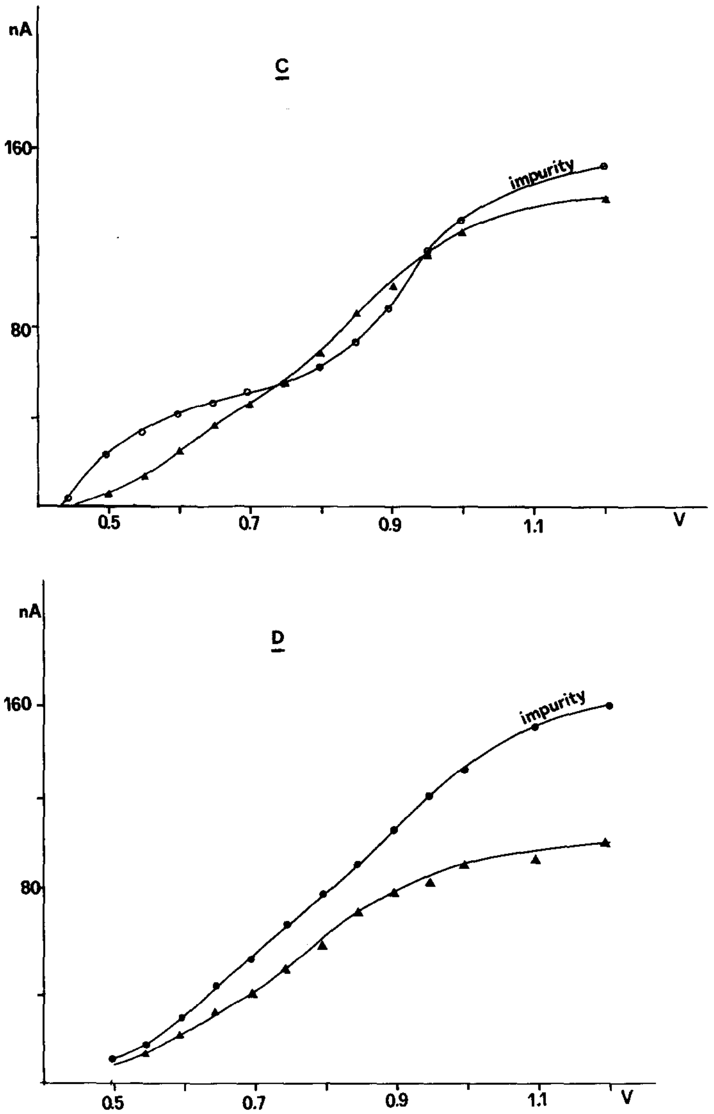

Fig. 5. Relationship between the detector response and applied potential: (A) (+)-catechin; (B) dimer B-2; (C) (▲) trimer C-1; (D) (▲) tetramer.

silage, obtained by simultaneous UV (280 nm) and electrochemical (+1.1 V) detection, is shown in Fig. 6. The importance of the determination of phenols in silages lies in the detrimental effect of these compounds in animal diets, *i.e.*, phenol-containing feedstuffs have a poor nutritive value. Maize should not contain large amounts of phenolics, and is thus a good material to use in detecting even small quantities of phenols in non-phenolic compounds. The ED trace appeared actually "cleaner" than the corresponding UV trace; the important absence of the two most conspicuous unknown peaks ($k' = 7$ and $k' = 23$) is explained as the UV detection

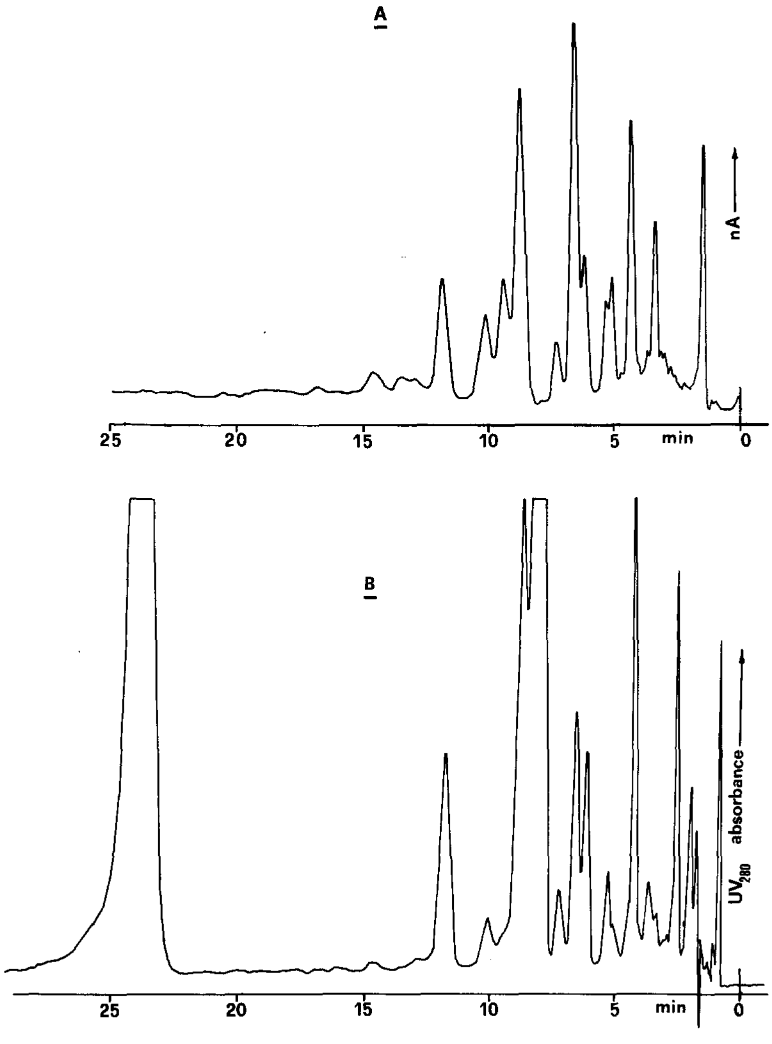

Fig. 6. Chromatograms of a maize silage extract: (A) ED trace (+1.10 V); (B) UV trace (280 nm). Chromatographic conditions as in Experimental section.

of non-oxidizable substances. Moreover, the peak ($k' = 2.3$) corresponding to catechin is definitely more evident in ED.

CONCLUSIONS

Electrochemical detection of naturally occurring polyphenols showed the following advantages over the more commonly adopted UV detection: (a) higher selectivity, particularly evident for catechin; (b) simplification of the chromatographic profile with regard to phenolic substances, owing to the insensitivity of ED towards other UV-absorbing unsaturated compounds (except aromatic amines).

ED was moreover not inferior to UV detection in showing an excellent linearity of response over the range of concentration studied. This makes possible external standardization even only at one point, and is particularly useful for routine quantitations. However, the current detector technology needs to be improved because the procedures (surface cleaning) and the precautions necessary to avoid electrode deactivation are still too time-consuming and laborious for daily routine analyses.

From the data presented, ED is particularly suited to polyphenolic compound detection. Its application to HPLC separations of extracts from a wide range of natural products is currently being tested in our laboratories.

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