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ELECTROCHEMICAL DETECTION OF CYANOGENIC GLYCOSIDES AFTER ENZYMATIC POST-COLUMN CLEAVAGE

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SUMMARY

Crude and partly purified extracts from *Helix pomatia* and linamarase from cassava were immobilized on columns packed with porous glass or silica and used as post-column reactors in the high-performance liquid chromatography of cyanogenic glycosides. Sodium hydroxide (2 *M*) was added to the flowstream after the enzyme-reactor resulting in the formation of cyanide, which was then detected at a silver electrode by an amperometric measurement at 0 V with reference to a silver–silver chloride electrode. The selective detection of cyanide allows measurements in a complex matrix. The response is linear and the detection limit is in the low picomole range.

INTRODUCTION

Cyanogenic glycosides are secondary plant products present in many plants consumed by humans or animals[1]. Methods for the separation and quantitative analysis of these compounds include thin-layer[2], gas[3–5], and liquid chromatography[6]. Selective detection requires a mass spectrometric measurement of the derivatized compounds[7,8] or hydrolysis of the glycoside and the intermediate cyanhydrin to yield an aldehyde and hydrogen cyanide, which in turn can be quantitatively determined. High-performance liquid chromatography (HPLC) using reversed-phase techniques is useful in the separation of the genuine cyanogenic glycosides, including epimeric pairs[6]. However, until now there has been no method available that permits selective detection of cyanogenic glycosides separated by HPLC. A potentially, useful method takes advantage of the selectivity provided by HPLC, enzymatic hydrolysis and electrochemical detection, previously demonstrated with phenolic glycosides[9]. The present paper is aimed at the development of a method for the determination of cyanogenic glycosides using a similar combination. The first step of the post-column reaction consists of the enzymatic hydrolysis outlined in eqn. 1 with cyanogenic glycosides as substrates for glycosidases. *Helix pomatia*, cassava and almond contain glycosidases that are potentially useful for this purpose. Hydrolysis leads to the formation of a cyanhydrin, which is easily hydrolysed further by alkali, yielding cyanide

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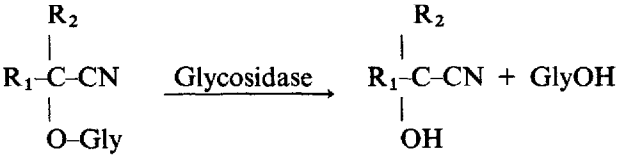
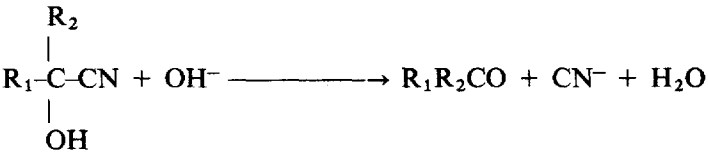
and an aldehyde or ketone (eqn. 2). As pointed out earlier[9], the use of amperometric detection of cyanide at a silver electrode might prove valuable in combination with post-column cleavage of cyanogenic glycosides. The electrode reaction shown in eqn. 3 consists of an oxidation of the silver to produce a soluble complex with cyanide[10,11].

Enzymatic reaction:



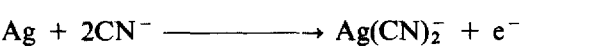
$$\begin{array}{l} R_2 \\ | \\ R_1\text{–}C\text{–}CN \\ | \\ O\text{–}Gly \end{array} \xrightarrow{\text{Glycosidase}} \begin{array}{l} R_2 \\ | \\ R_1\text{–}C\text{–}CN \\ | \\ OH \end{array} + GlyOH \qquad (1)$$

Base-catalysed cleavage:



$$\begin{array}{l} R_2 \\ | \\ R_1\text{–}C\text{–}CN \\ | \\ OH \end{array} + OH^- \longrightarrow R_1R_2CO + CN^- + H_2O \qquad (2)$$

Anodic reaction:



$$Ag + 2CN^- \longrightarrow Ag(CN)_2^- + e^- \qquad (3)$$

EXPERIMENTAL

Chemicals

Amygdalin was purchased from Fluka (Switzerland), prunasin was from Carl Roth (Karlsruhe, F.R.G.) and linamarin was from Calbiochem (san Diego, CA, U.S.A.). Linamarase (from cassava) was purchased from BDH (Poole, U.K.). Mandelonitrile was prepared from benzaldehyde and potassium cyanide[12]. *p*-Nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-glucuronide and glucuronidase (type 2 from *H. pomatia*) were obtained from Sigma (St. Louis, MO, U.S.A.).

Controlled-pore glass (CPG) with a pore size of 550 Å was purchased from Pierce (Rockford, IL, U.S.A.) and LiChrospher Si 500 from Merck (Darmstadt, F.R.G.) was a gift from Silvaco (Copenhagen, Denmark).

Enzyme reactors

Aminopropyl-CPG was prepared from 3-aminopropyl triethoxysilane (EGA-Chemie, Brenz, F.R.G.) and CPG by refluxing in toluene[13]. The reactors were packed and loaded by a new method[14], as follows: the dry material was poured into the top of a vertical steel column (50 × 3 mm I.D.) with one Swagelok end-fitting connected, and the material was packed by gentle tapping of the column, which was then closed and connected to the injection valve. Then 5 × 1 ml of 2.5% glutaraldehyde (art. 4239, Merck) in water was introduced through a 1-ml loop while a flow of 1 ml/min of distilled water was maintained through the column. After *ca.* 10 min the loop was

changed to 100 μl and 3 × 100 μl of the enzyme solution was injected now using a flow of 0.1 ml/min. The eluate was collected and the activity was measured with *p*-nitrophenyl-glucoside and *p*-nitrophenyl-glucuronide as substrates.

LiChrospher Si 500 was similarly derivatized and packed in a column by use of the dilute slurry technique. Immobilization was carried out as above.

Instrumentation

The chromatographic system (Fig. 1) consisted of an LDC Constametric Model III pump, equipped with a Rheodyne 7125 valve, and a 25 × 4.6 mm I.D. Spherisorb S5-C8 analytical column. The detection system was made up of SSI T-pieces, 0.15 mm I.D. PTFE tubing, the enzyme reactor and a Metrohm 656/641 electrochemical detector. The electrodes of the wall-jet cell consisted of a silver–silver chloride–3 *M* potassium chloride reference electrode, a glassy carbon auxiliary electrode, and a silver working electrode. Sodium hydroxide (2 *M*) was fed into the flowstream by means of a thick-walled 1-l glass bottle (Gerhardt) pressurized with nitrogen (4 atm) and fitted with PTFE tubing inserted through a rubber stopper. Omnifit unions and valves were used for connecting thick and thin tubing and for venting purposes, respectively. A flow-rate of *ca.* 0.2 ml/min of sodium hydroxide (2 *M*) was maintained.

Chromatograms were recorded on an Omniscribe recorder (Houston Instru-

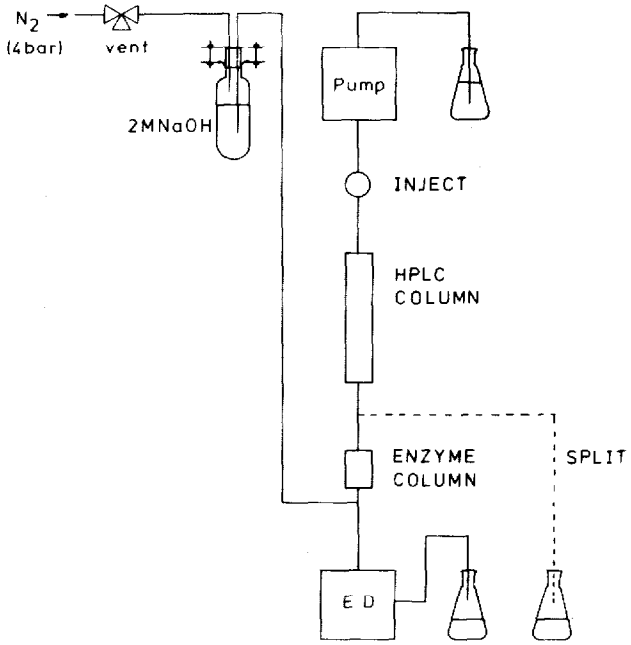


Fig. 1. HPLC system used for detection of cyanogenic glycosides. Post-column cleavage was achieved by use of immobilized glycosidases packed in columns (50 × 3 mm I.D.). Cyanohydrins formed by the enzymatic reaction were further degraded to cyanide by alkali introduced through a T-piece from a pressurised reservoir of 2 *M* sodium hydroxide. Prolonged residence time in the enzyme reactors could be achieved by introducing a split between the chromatographic column and the enzyme reactor. This was found useful in detection of substrates with low enzyme affinity. Cyanide was eventually detected at a silver electrode by an amperometric measurement.

ments) and integration was performed with an SP-minigrator or an SP-4270 integrator (Spectra-Physics).

Procedures

Enzyme activities of glucosidases were measured before immobilization, and residual activities of eluates from the enzyme reactors were measured after immobilization. A 500-μl volume of *p*-nitrophenyl-β-D-glucoside or *p*-nitrophenyl-β-D-glucuronide (5–10 m*M*) in 0.05 *M* phosphate buffer, pH 5.0, and 500 μl of the enzyme solution were thermostatted at 37.0°C and mixed. After 15 min 3.0 ml of 0.22 *M* glycine–sodium chloride buffer, pH 11, was added and the absorbance measured at 420 nm. The reference solution was prepared in parallel using distilled water instead of the enzyme solution. The standard used was 0.04 m*M* *p*-nitrophenol in glycine–sodium chloride buffer. If more than 5% of the substrate was hydrolysed the measurement was repeated after dilution of the enzyme.

Qualitative spot tests based on the hydrogen cyanide reaction with picric acid[15] were used in the purification of *H. pomatia* juice. Linamarin, prunasin, and amygdalin were used as substrates.

Efficiency of enzyme reactors. Volumes of 20 μl of 0.04–0.5 m*M* solutions were injected using the chromatographic system in Fig. 1, omitting the detector. A fraction of the eluate was collected at the expected retention volume of the respective glycosides. The eluate was lyophilized and redissolved in the mobile phase (200 μl), and 20 μl were injected again, this time with the detector connected.

Purification of glycosidases. A method similar to that of Got and Marnay[16] was used in preliminary experiments. Crude *H. pomatia* juice (1 ml) was applied to a column (100 × 2.5 cm I.D.) containing Sephadex G-200 and eluted with 0.05 *M* phosphate buffer, pH 5.0. Fractions of 10 ml were collected and enzyme activities were measured using linamarin, amygdalin, prunasin, *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-glucuronide as substrates. The elution pattern essentially followed that observed earlier[16], showing the presence of two glycosidases. Further experiments were performed, using a 40 × 2.5 cm I.D. column. Fractions number 10–24 were pooled and dialysed overnight and finally freeze-dried. The dry material amounted to *ca.* 50 mg.

RESULTS AND DISCUSSION

Evaluation of the silver electrode

Injection of cyanide solutions. The influence of pH, the presence of methanol in the mobile phase, and the potential applied to the silver electrode were studied. For this purpose the chromatographic system (Fig. 1) was changed by connecting the pump, the injection valve, the T-piece for inlet of alkali and the electrochemical detector in series. The pH dependence of the response is shown in Fig. 2. A solution of 0.02 m*M* potassium cyanide was injected through the 20-μl loop and the peak height was measured for various pH values using appropriate 0.05 *M* phosphate buffers as mobile phase. A pH of 13 was reached by addition of alkali. The precence of 50% methanol in the mobile phase (pH 9) had no visible effect on the detector response. The optimum potential for detection of cyanide was found to be 0 V, in accordance with earlier findings[10,11], and detection of 2 pmol of cyanide was easily attained under these conditions.

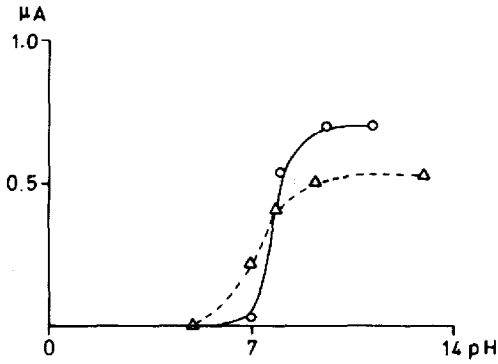


Fig. 2. Relationship between the pH of the eluent and the current response. To obtain the current response for cyanide (triangles), the chromatographic column and the enzyme reactor were disconnected and replaced by PTFE tubing; mobile phase, 0.05 *M* phosphate; flow-rate, 1 ml/min. The current response for mandelonitrile (dots) was obtained using the chromatographic system in Fig. 1 without the enzyme reactor; mobile phase, 15% methanol in 0.05 *M* phosphate, pH 5; flow-rate, 1 ml/min; the pH of the eluate from the detector was adjusted with alkali (0.2 *M*) introduced between the enzyme column and the detector.

Injection of other compounds. The detector responded to mandelonitrile under alkaline conditions, whereas the other cleavage products formed by enzymatic hydrolysis in the reactor, *e.g.* benzaldehyde or glucose, could not be detected. Injection of sulphur compounds, such as cysteine, glutathione, and sodium diethyl dithiocarbamate, revealed that the silver electrode was sensitive to this type of compound also.

Optimum conditions for the detection of mandelonitrile were found using the chromatographic system (Fig. 1) without the enzyme reactor. Impurities in the sample (benzaldehyde and free cyanide) could thus be separated from the target compound. The mobile phase, 15% methanol in 0.05 *M* phosphate (pH 7.0), was mixed with various proportions of alkali (introduced through the T-piece), and the resulting pH of the mixture was measured. The pH dependence of the response to mandelonitrile is shown in Fig. 2. Although a plateau was reached at a pH between 9 and 10, a pH of *ca.* 13 was chosen for further development of the chromatographic system. This choice was based on the proton dissociation constant (pK_a 10.7) for mandelonitrile, the rate constant, and the apparent equilibrium constant for the hydrolysis of this compound[17]. The pH dependence of the apparent equilibrium constant is given by:

$$K_{app} = 236\ (1 - \alpha_{CN}) + 5.5\alpha_{CN} \tag{4}$$

were

$$\alpha_{CN} = 1/(1 - a_H/K_a) \tag{5}$$

and a_H and K_a are the proton activity and the dissociation constant for hydrogen cyanide, respectively. At pH 13 and pK 9.10, a 1 mM solution was calculated to be hydrolysed to an extent of 99%. The rate of hydrolysis, given by eqn. 6,

$$\text{rate} = 2.5 \cdot 10^{-4}\ [>C(OH)CN]\ [OH^-] \tag{6}$$

suggests a half-life of 0.05 sec at pH 13. The tubing between the T-piece (where alkali was introduced) and the detector contained a volume of 25 μl. For a flow-rate of 1 ml/min through the HPLC column and additional 0.2 ml/min of alkali, the residence time is thus 1.25 sec, which is 25 times the half-life. Therefore hydrolysis is complete at pH 13.

Enzyme reactors

CPG and porous silica (Lichrospher Si 500) served as rigid backbones for the immobilized enzymes. The two materials have similar characteristics with regard to void volume, dispersion and loading capability. However, the backpressure observed using Lichospher was considerably higher (*ca.* 300 p.s.i.) than that observed with CPG, which was negligible.

The enzymes from *H. pomatia* used in this work were partly purified by gel filtration on Sephadex G-200, yielding material with a specific activity increased by three or four times. It is known[16] that *H. pomatia* contains two glycosidases with affinity for both galactosides and glucosides. In preliminary experiments it was found that the cyanogenic glycosides had different affinities towards the two enzymes. In the present investigation the enzymes were pooled for use in enzyme reactors, thereby covering a broader spectrum of substrates. Also, glucuronidase was present after gel filtration. The amounts of enzyme loaded on the columns were in the range 10–15 mg with corresponding activities towards *p*-nitrophenyl-β-D-glucoside in the range 10–13 I.U. for *H. pomatia* enzymes and 21 I.U. for linamarase (Table I). Attempts at the direct measurement of the enzyme activity present in the reactors by the flow-through technique[9] were abandoned because a high percentage of the substrate was converted at moderate flow-rates. The use of a high flow-rate was not possible with reactors containing Lichrospher Si 500, owing to the high backpressure experienced with this material. The pH optimum of the enzymes is *ca.* 5 and the mobile phase

TABLE I

LOADING OF PACKED BED REACTORS WITH CRUDE AND PARTLY PURIFIED GLYCOSIDASES FROM *HELIX POMATIA* AND CASSAVA

Reactor	*Substrate*	*Enzyme activity (I.U.)**
Crude enzyme from *H. pomatia*/CPG	*p*-Nitrophenyl-β-D-glucoside	(3)
	p-Nitrophenyl-β-D-glucuronide	(0.5)
Purified enzyme from *H. pomatia*/CPG	*p*-Nitrophenyl-β-D-glucoside	10
	p-Nitrophenyl-β-D-glucuronide	2
Purified enzyme from *H. pomatia*/LiChrospher Si 500	*p*-Nitrophenyl-β-D-glucoside	13
	p-Nitrophenyl-β-D-glucuronide	2
Purified enzyme from cassave/CPG	*p*-Nitrophenyl-β-D-glucoside	21

* Numbers in parentheses represent measurements obtained by flow-through technique[9].

was prepared accordingly, using 0.05 *M* phosphate. A content of 15% methanol in the mobile phase resulted in sufficient selectivity with regard to the separation of pairs of some epimers, but a lower content of methanol was necessary in a few applications[18]. The temperature of the reactors was maintained at 37°C except when the efficiency of the enzyme was evaluated.

Efficiency of the enzymatic reaction

The reactors could be used for several months before ageing of the enzymes caused a drop in response. For quantitative purposes complete hydrolysis is not imperative if the substrate is present in amounts well above the theoretical detection limit and the individual standard is available. When using the present detection system in applications where reference compounds are not available complete hydrolysis is essential. One of the advantages of this latter method arises from the fact that detection is based on a common product (cyanide), which will result in the same coulometric yield for all cyanogenic compounds. Accordingly, determinations should be based on area measurements using a single internal or external standard belonging to this type of compound.

A number of possibilities for determination of the efficiency of hydrolysis were considered. The parameters that could be affected in such a way that increased hydrolysis might take place were: temperature (increase) flow-rate (decrease), and reactor volume (increase). The last possibility was excluded because additional peak broadening would result in lower resolution. Triplicate injections at 30, 35 and 40°C showed similar detector responses, indicating that complete hydrolysis was taking place in this range of temperature. The simplest method of testing whether complete hydrolysis was achieved, was to change the flow-rate, either by introducing a split or

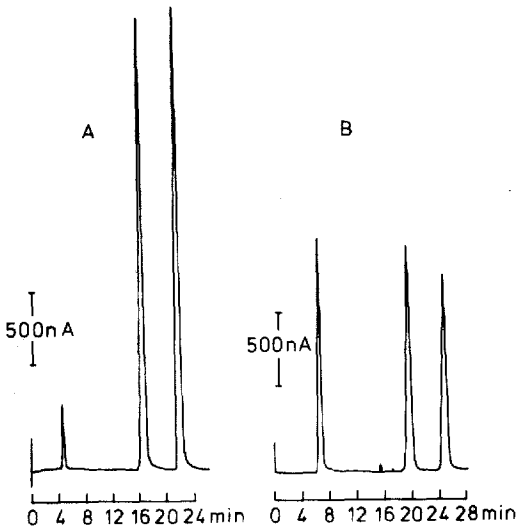


Fig. 3. (A) Separation of linamarin, amygdalin, and prunasin. Mobile phase, 15% methanol in 0.05 *M* phosphate, pH 5.0; flow-rate, 1 ml/min; working potential, 0.0 V. (B) A split was introduced between the chromatographic column and the enzyme reactor thereby reducing the flow-rate through the enzyme reactor to 0.25 ml/min. This method is suitable for enhancement of the signal from linamarin and other substrates that have low affinity for the enzyme.

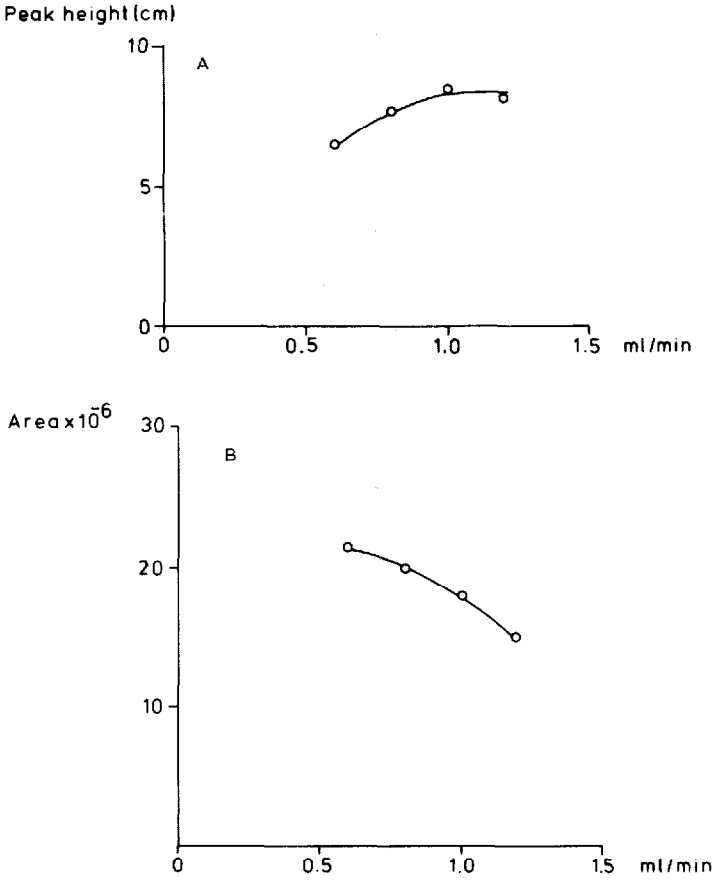


Fig. 4. Relationship between peak height (A) or area (B) and flow-rate. Linamarin was injected using the chromatographic system in Fig. 1. The enzyme reactor contained linamarase. Mobile phase, 15% methanol in 0.05 *M* phosphate, pH 5; working potential, 0.0 V.

more readily by using a lower pump speed. The effect was clearly demonstrated with linamarin, which was only partly hydrolysed using enzymes from *H. pomatia.* Fig. 3 shows the enhancement of the response from linamarin by introduction of a split. A similar but less dramatic effect was observed using a lower pump speed. If, however, complete hydrolysis was accomplished, a change of the flow-rate had little effect on the peak height, as demonstrated with linamarin and linamarase (Fig. 4A). In this context it was obvious that measurements of areas were less suitable as a test parameter, because the coulometric yield of an amperometric detector increases with decreasing flow-rate (Fig. 4B). The efficiency of hydrolysis of concentrated solutions (0.04–2 m*M*) was also measured. Prunasin and amygdalin were completely hydrolysed whereas only 20–40% of linamarin was converted in the enzyme reactor containing purified material from *H.* pomatia. Linamarase from cassava, by contrast, hydrolysed linamarin completely, leaving prunasin and amygdalin unaffected.

Linearity, detection limit, and reproducibility

Linear standard curves were obtained in the range from 1 m*M* to 1 μM for all the substrates tested using either peak height or area. The detection limit was *ca.* 20 pmol (signal-to-noise ratio 3) for standard solutions, the limiting factor being set by pump pulsations.

Response factors (area counts/mol/l) for various dilutions of a mixture of amygdalin and prunasin were calculated at several concentration levels (from 0.7 m*M* to 5 μM). Although the precision was poor, similar values (area counts/mol/l $\pm$ S.D., $n = 10$) were obtained for amygdalin [$(5.1 \pm 0.9) \cdot 10^{10}$] and prunasin [$(5.0 \pm 1.1) \cdot 10^{10}$] at all levels, indicating complete hydrolysis of the two compounds. Not surprisingly, much better precision was found with relative response factors. The experimental value (prunasin/amygdalin) of 1.08 $\pm$ 0.09, $n = 10$, was close to the expected value of 1.05. Repeated injections of the same standard gave a relative standard deviation of 4% ($n = 9$).

Applications

The present method was primarily intended for determination of cyanogenic glycosides in plant material[18]. However, the method could also be used for detection of cyanogenic glycosides in serum and urine. Fig. 5 shows the chromatograms of human urine and linamarine added to human urine. The presence of glucuronidase in enzyme reactors employing *H. pomatia* opens up the possibility for selective detection of mandelonitrile glucuronide, currently being investigated as a potential anticancer drug.

Further applications might include the determination of cyanide or cyanhydrins in complex mixtures requiring a separation step (HPLC). Recently[19], determination of cyanide in waste water was performed using a procedure in which the

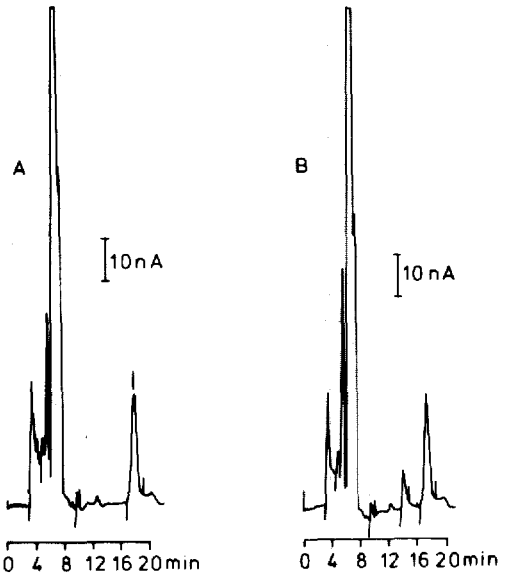


Fig. 5. Chromatograms of human urine (A) and human urine containing 5.5 μM of linamarine (B). Mobile phase, 0.05 *M* phosphate, pH 5; flow-rates, 1 ml/min; working potential, 0.0 V.

cyanide was converted into cyanhydrin, which was then measured by gas chromatography. A similar derivative might be used for liquid chromatography employing post-column cleavage by alkali and electrochemical detection.

CONCLUSION

A reversed-phase chromatographic system combined with a selective and sensitive detection method has been developed. The influence of a number of parameters on the detection of cyanogenic glycosides has been studied and the optimum conditions found. The system is potentially useful for both qualitative and quantitative applications. The long-term stability and reliability of the system are good. Periodic evaluation of the enzyme reactors with regard to efficiency of hydrolysis can easily be carried out by decreasing the flow-rate followed by injection of the sample. An increased peak height would then indicate incomplete hydrolysis at the normal flow-rate, necessitating renewal of the enzyme reactor.

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