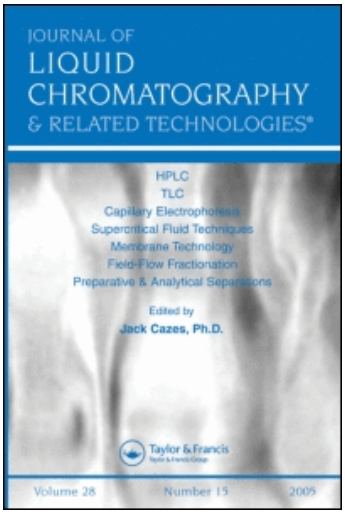

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High Performance Liquid Phase Separation of Glycosides. I. Reversed Phase Chromatography of Cyanogenic Glycosides with UV and Pulsed Amperometric Detection

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HIGH PERFORMANCE LIQUID PHASE SEPARATION OF GLYCOSIDES. I. REVERSED PHASE CHROMATOGRAPHY OF CYANOGENIC GLYCOSIDES WITH UV AND PULSED AMPEROMETRIC DETECTION

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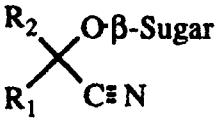
ABSTRACT

High performance liquid chromatography procedures based on reversed phase chromatography (RPC) using microparticulate octadecylsilica columns were introduced for the separation and detection of some representative cyanogenic glycosides and their degradation products. Pulsed amperometric detection (PAD) provided relatively low detection limits (10^{-5}–10^{-7} M) for the cyanogenic glycosides and permitted the detection of those lacking a chromophore in their structures (e.g., linamarin) which could not be detected in the UV even at low wavelength. In addition, the PAD was a more selective method of detection when compared to UV at 200 nm, a wavelength at which the molar absorptivity and in turn the detection sensitivity were relatively high for the chromophoric cyanogenic glycosides. However, and in the presence of acetonitrile in the eluent, the detector response in PAD was linear in concentration range over

2 to 3 orders of magnitude as opposed to 4 to 5 orders of magnitude in the UV. Finally, RPC proved very useful in monitoring the rate of hydrolysis of mandelonitrile to benzaldehyde, which is a degradation product of the cyanogenic glycosides amygdalin and prunasin. This hydrolysis was found to be a first order reaction.

INTRODUCTION

Many plant derived foodstuffs or tissues release HCN by the hydrolysis of secondary products, namely the cyanogenic glycosides and cyanolipids.[1] This ability of releasing HCN is know as cyanogenesis. Hydrolysis generally follows the disruption of cyanophoric tissues either by crushing, mastication or fungal injury, whereby endogenous hydrolases are allowed access to cyanogenic glycosides or cyanolipids, whose rapid catabolism to the lethal metabolite HCN is promoted. Cyanogenic glycosides are naturally occurring toxins that have been determined to be present in more than 2050 different plants distributed throughout 110 different families[2] that may be used as food sources by humans and domestic animals. There are, however, only approximately 26 known cyanogenic glycosides, all of which are derived from α-hydroxynitriles.[1] The general formula for cyanogenic glycosides is as follows



where the sugar moiety is usually a glucose and less often a gentiobiose, vicianose or primeverose. R_1 is either an aliphatic or aromatic group while R_2 is an H in a majority of the cyanogenic glycosides. When the sugar of the glycoside is removed, both HCN and a carbonyl are released.[3] Many of these cyanogenic glycosides are ingested by animals and also are nutritionally useful. Toxicity is the result of the release of HCN upon hydrolysis or enzymatic action on the glycoside.[4]

High performance liquid chromatography (HPLC) has been applied to the isolation and determination of cyanogenic glycosides including the use of hydroxyapatite chromatography,[5] normal-phase chromatography using silica gel columns[6] and reversed phase chromatography using primarily octadecylsilica (C_{18}) columns[7,8] (see Ref. 9 for a discussion) and to a lesser extent octylsilica (C_8) columns.[10,11] In these HPLC systems, UV detectors have been used for the detection of chromophoric cyanogenic glycosides while

refractive index (RI) detectors have been employed for detecting non absorbing cyanogenic glycosides. While UV detectors have provided an adequate detection sensitivity, RI detectors have been known for their low sensitivity and incompatibility with gradient elution. To overcome the difficulties associated with the detection of cyanogenic glycosides that lack strong chromophores in their structures, electrochemical detection has been attempted.[10] However, in that work,[10] a post-column enzymatic reactor was needed for the cleavage of the cyanogenic glycosides to form HCN, which was followed by converting the liberated HCN to CN^- by the addition of NaOH, and the ultimate cleavage product CN^- was then detected electrochemically. Thus, there is a need for a method for determining weak chromophore-containing cyanogenic glycosides that is simple and reproducible.

This report is concerned with the evaluation of pulsed amperometric detection (PAD) in the detection of cyanogenic glycosides, and the results are compared with UV detection. To the best of our knowledge, PAD has not been yet exploited in the determination of cyanogenic glycosides, despite the fact that PAD has been found to be quite useful for the detection of analytes that do not possess a strong chromophore.[12] With PAD, there is no need for post-column modification of the separated analytes; thus, providing an easily managed method of detection. In addition, PAD is a very simple, sensitive, and selective method for the detection of a variety of different analytes, such as carbohydrates, alcohols, alkanolamines, and sulfur-containing compounds.[13-15]

EXPERIMENTAL

Instruments and Columns

The liquid chromatograph was assembled from (i) an LDC Analytical (Riviera Beach, FL, USA) solvent delivery system comprising a ConstaMetric 3500 pump with a gradient programmer, which also controlled a ConstaMetric Model III pump, (ii) a Model 7125 sample injector from Rheodyne (Cotati, CA, USA), (iii) a variable wavelength detector Model SpectroMonitor 3100 from LDC Analytical, (iv) a pulsed amperometric detector (PAD) from Dionex (Sunnyvale, CA, USA), and (v) a Shimadzu (Columbia, MD, USA) integrator Model C-R5A Chromatopac.

For UV monitoring of the column effluent, the detector was set at 200 nm with a response time of 0.50 sec. For pulsed amperometric detection of the solutes, 0.50 M NaOH was added to the column effluent at a flow rate of 0.7–0.8 mL/min. This post-column addition was accomplished via a T-connector

which allowed the mixing of the column effluent with the stream of the NaOH solution that was delivered by a nitrogen pressurized reservoir containing the NaOH solution. The two liquids (i.e., the column effluent and NaOH solution) were further mixed in a post-column reaction coil packed bead before entering the detection cell of the PAD. The pH of the combined streams of column effluent and post-column addition of NaOH yielded a solution whose pH was found to be 12.7.

The PAD is equipped with a gold working electrode, Ag/AgCl reference electrode and a solvent compatible PAD-2 cell. The PAD settings for pulse potentials and duration times were E_1 = 0.00 V, t_1 = 240 ms; E_2 = 0.60 V, t_2 = 60 ms; E_3 = -0.80 V, t_3 = 60 ms.

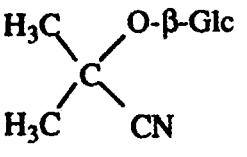
The reversed phase packing consisted of Microsorb-MV C18, 5 μm, 100 Å from Rainin (Woburn, MA, USA), and was supplied in packed columns of dimensions of 250 x 4.6 mm or 150 x 4.6 mm.

The UV spectra of the various cyanogenic glycosides were obtained with a diode array spectrophotometer from Hewlett Packard (Waldbronn, Germany) Model 8452A which was utilized over the spectral range from 190 nm to 400 nm with an integration time of 1.0 sec.

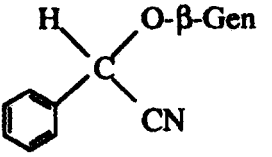
Reagents and Materials

Acetonitrile of HPLC grade as well as high purity UV was obtained from J.T. Baker (Phillipsburg, NJ, USA) and from Baxter Diagnostics Inc. (McGraw Park, IL, USA), respectively. Sodium hydroxide solution (50% w/w) was purchased from Fisher (Pittsburgh, PA, USA), and purified water was prepared with a Barnstead Nanopure ultrapure water system. Benzaldehyde was purchased from Mallinckrodt (St. Louis, MO, USA).

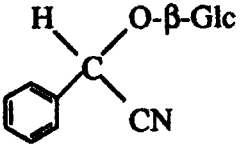
The cyanogenic glycosides linamarin (phaseolunatin), prunasin (D-mandelonitrile-β-D-glucoside) and D-amygdalin (D-mandelonitrile 6-O-β-D-glucosido-β-D-glucoside) from apricot kernels as well as their degradation product DL-mandelonitrile (α-hydroxyphenylacetonitrile) were purchased from Sigma (St. Louis, MO, USA). The structures of the three cyanogenic glycosides and mandelonitrile (the degradation product of prunasin and amygdalin) are shown below.



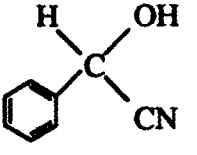
Linamarin



Amygdalin



Prunasin



Mandelonitrile

where Glc = glucose and Gen = gentiobiose. The standard cyanogenic glycoside samples were dissolved in water, while the standard mandelonitrile was dissolved in acetonitrile. Eluents were filtered, sonicated and degassed with ultra pure grade helium.

RESULTS AND DISCUSSION

Two different methods of detection were utilized and compared in this study, namely UV and pulsed amperometric detection (PAD). The solutes were chromatographed by either gradient or isocratic elution.

Figure 1a and b shows typical chromatograms obtained by 10 and 6 min linear gradient elution, respectively, at increasing acetonitrile concentration in the eluent using PAD for sensing the column effluent. As can be seen in Figure 1a and b, the four solutes are fully resolved into narrow peaks. In contrast, Figure 2 shows a typical isocratic separation using a mobile phase of 82:18 water/acetonitrile (v/v). It should be noted that peak No. 4 in Figures 1a and 2 is believed to be the peak of benzaldehyde which was assigned (see below) by studying the degradation of mandelonitrile. Although the same analysis time is achieved in both cases, gradient elution provided higher separation efficiency (compare Figures 1a and 2). In addition, linamarin which is not strongly retained eluted very close to the dead volume where other contaminants of the sample emerged; see Figure 2.

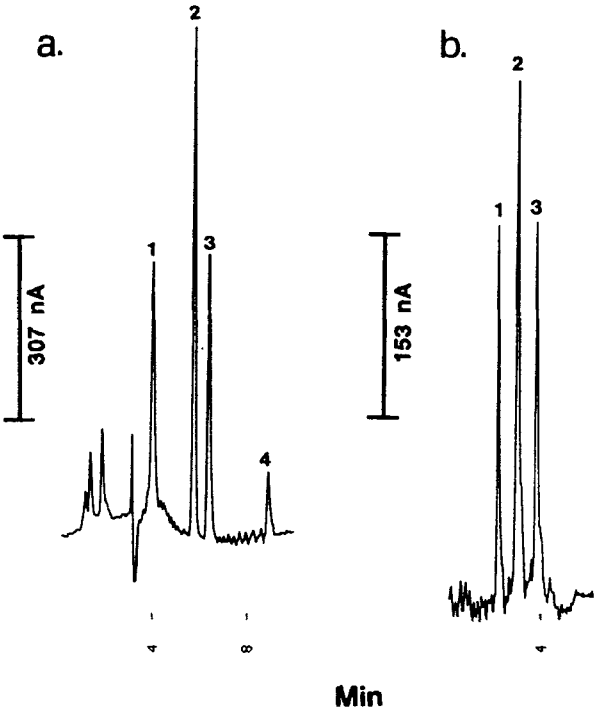


Figure 1. Typical chromatograms of cyanogenic glycosides obtained by gradient elution using PAD. Column, Microsorb MV C18, 15 cm x 4.6 mm i.d. in (a), 25 cm x 4.6 mm i.d. in (b); (a) linear gradient in 10 min from 0.0 to 60% (v/v) acetonitrile in water; (b) linear gradient in 6 min from 18.0 to 60% (v/v) acetonitrile in water; flow rate, 1.5 mL/min.
Peaks: 1, linamarin; 2, amygdalin; 3, prunasin; 4, benzaldehyde.

To select the appropriate wavelength for UV detection, spectral data were obtained for each of the cyanogenic glycosides studied as well as for the degradation products in the range of 190 to 400 nm. With the exception of linamarin, each of the solutes exhibited two distinct absorption maxima, see Table 1. The highest absorption maxima were at 192 nm for both amygdalin and prunasin. The other less pronounced absorption maxima were at 206 nm and 208 nm for amygdalin and prunasin, respectively. While prunasin showed a small maximum at 262 nm, amygdalin did not show a distinct maximum at this wavelength and the molar absorptivity was relatively low, see Table 1.

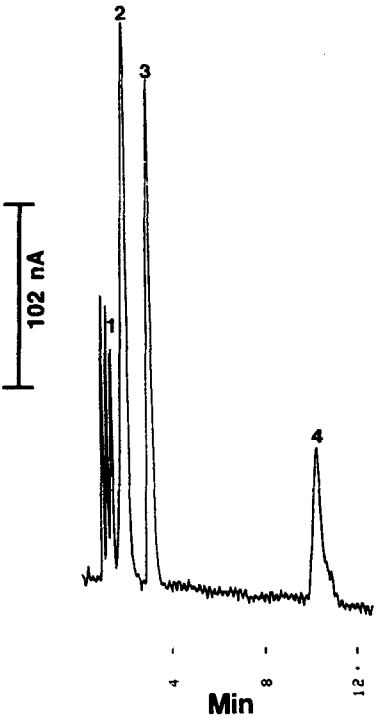


Figure 2. Typical chromatogram of cyanogenic glycosides obtained by isocratic elution using PAD. Column, Microsorb MV C18, 15 cm x 4.6 mm i.d.; mobile phase, water at 18% acetonitrile (v/v); flow rate, 1.5 mL/min. Peaks as in Fig. 1.

The intermediate and degradation products of both amygdalin and prunasin, i.e., mandelonitrile and benzaldehyde, respectively, showed the highest absorption maxima at 196 and less pronounced maxima at 251 nm. Based on these spectral data, a 200 nm wavelength was selected for the detection of the four UV detectable solutes.

Returning to Table 1, it is clear that adding a sugar residue to mandelonitrile to yield prunasin decreased slightly the molar absorptivity and shifted the less pronounced absorption maximum to lower wavelength (i.e., from 251 nm to 208). Adding another glucose moiety to prunasin to yield amygdalin did not result in a significant change in the spectral maxima and molar absorptivity, see Table 1.

Table 1

Absorption Maxima and Molar Absorpitivity

Cyanogenic	λ_1 (nm)/ε_1 ($M^{-1}cm^{-1}$)	λ_2 (nm)/ε_2 ($M^{-1}cm^{-1}$)	λ_3 (nm)/ε_3 ($M^{-1}cm^{-1}$)
Linamarin	200 / 6 420		
Amygdalin	192 / 37 280	206 / 5 700	262 / 716
Prunasin	192 / 35 320	208 / 7 355	262 / 3 807
Mandelonitrile	196 / 42 435	251 / 10 472	
Benzaldehyde	196 / 45 555	251 / 10 041	

As just stated above, only four solutes could be detected at 200 nm, namely amygdalin, prunasin, mandelonitrile and benzaldehyde. Figure 3 illustrates a typical UV chromatogram obtained with a 5 min linear gradient at increasing acetonitrile concentration in the eluent. The starting eluent contained 30% (v/v) acetonitrile to affect the elution and separation of the three solutes with the gradient time selected. This high organic modifier was necessary since increasing gradient steepness (from 3 to 12% v/v acetonitrile/min) by decreasing the gradient time while maintaining the composition of the starting eluent the same (i.e., 0% v/v acetonitrile) did not allow the elution of all analytes. As with the PAD detection, when eluted isocratically, the peaks were broader, and in addition the analysis time was doubled (results not shown).

Returning to Figures 1a, 2 and 3, as mentioned above, the last eluting peak was the degradation product of mandelonitrile, namely benzaldehyde. The rationale behind studying the chromatographic behavior of mandelonitrile resides in the fact that mandelonitrile is a degradation product of prunasin and amygdalin. In fact, in cyanophoric plants, there exists enzymes which are specific for the β-glycosidic linkage.

Generally, when the plant tissue is crushed or otherwise disrupted, the glycoside comes into intimate contact with β-glucosidases which hydrolyze the cyanogenic glycoside producing D-glucose and the respective α-hydroxynitrile (see also **INTRODUCTION**). The latter may dissociate spontaneously, releasing HCN and the corresponding aldehyde or ketone. However, cyanogenic tissues generally possess a second enzyme, an hydroxynitrile lyase, which catalyzes the dissociation of the cyanohydrin.[9]

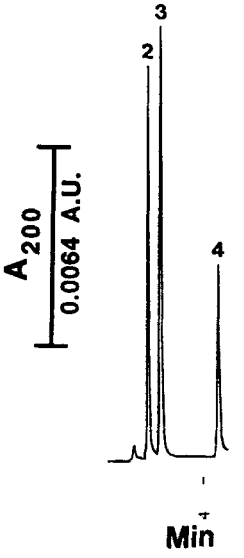
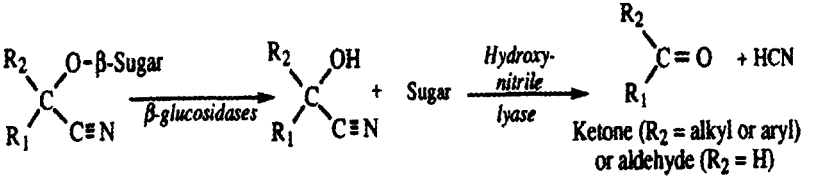


Figure 3. Typical chromatogram separation of cyanogenic glycosides obtained by gradient elution using UV detection at 200 nm. Column, Microsorb MV C18, 25 cm x 4.6 mm i.d.; linear gradient of 5 min from 30.0 to 60.0% (v/v) acetonitrile in water; flow rate, 1.5 mL/min. Peaks as in Fig. 1.



Over time, and as it has been reported in the literature,[10] mandelonitrile breaks down due to hydrolysis to form benzaldehyde and HCN. In fact, whenever the mandelonitrile sample solution was freshly prepared, two peaks were consistently associated with the solution of mandelonitrile. However, over time the apparent ratio of the peaks changed reflecting the appearance of the product and disappearance of the analyte. To determine the time course and conditions required for total breakdown of mandelonitrile, a series of injections was undertaken using isocratic elution with a mobile phase at 27% (v/v) acetonitrile. Figure 4 shows three chromatograms corresponding to the initial

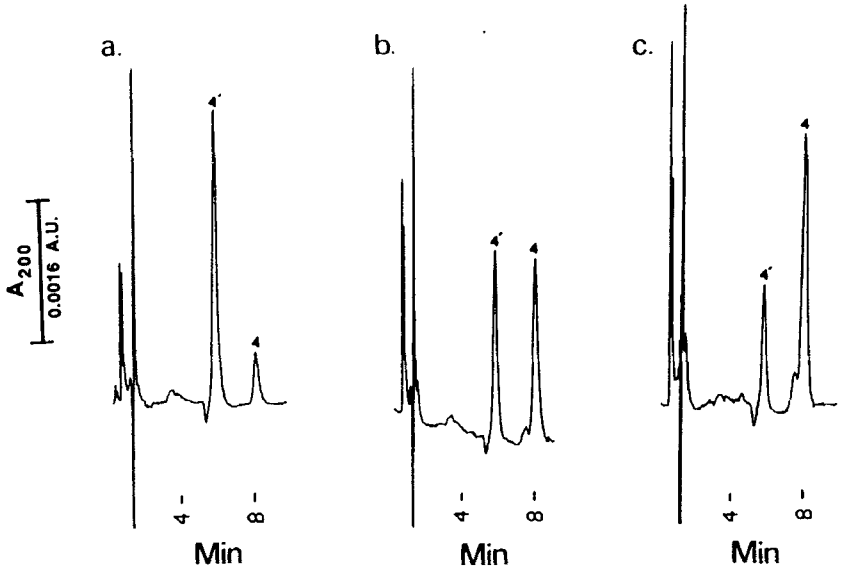


Figure 4. Typical chromatograms for the degradation of mandelonitrile to the product benzaldehyde obtained by isocratic elution using UV detection at 200 nm. (a) Initial preparation; (b) 1 hour after preparation; (c) 3 hours and 30 minutes after preparation. Column, Microsorb MV C18, 15 cm x 4.6 mm i.d.; mobile phase, water at 27% (v/v) acetonitrile; flow rate, 1.5 mL/min. Peaks: 4', mandelonitrile; 4, benzaldehyde.

preparation, 1 hour after preparation, and after 3 hours and 30 min of standing at room temperature. Figure 5a gives the time course of the degradation of mandelonitrile, expressed in terms of peak height ratio (mandelonitrile/benzaldehyde) vs. time. To determine the order of the hydrolysis reaction, Figure 5b shows the dependence of the natural logarithm of the peak height of mandelonitrile, which reflects the natural logarithm of mandelonitrile concentration, on time. The plot is linear indicating a first-order reaction.

Using isocratic elution, the retention of glycosides under investigation showed strong dependence on the percent acetonitrile in the eluent. Plots of logarithmic capacity factor of the solutes under investigation vs. percent acetonitrile (in the percent range from 0 to 25%) were linear with R values greater than 0.993 (results not shown), thus indicating a reversed phase chromatography behavior.

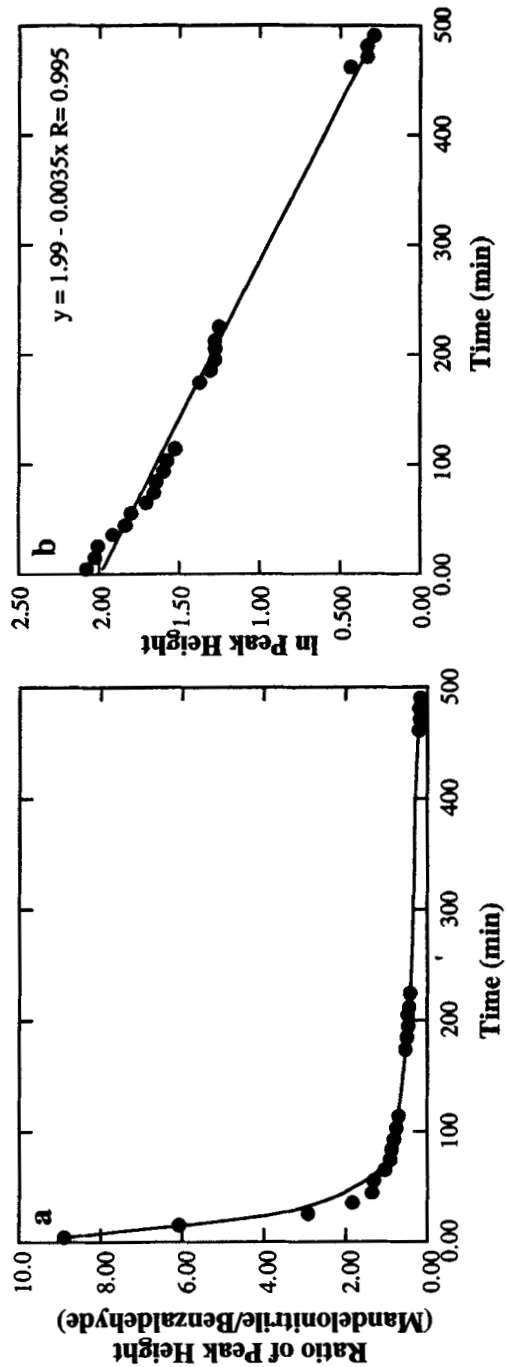


Figure 5. (a) Plot of the ratio of mandelonitrile/benzaldehyde peak height *versus* time; (b) plot of logarithmic peak height of mandelonitrile *versus* time. Conditions as in Fig. 4.

It should be noted that with PAD detection, as the percent acetonitrile in the eluent was increased, baseline noise increased. It has been reported that acetonitrile can interfere with the detector response by adsorbing to the surface of the noble-metal electrode.[16] Therefore, detection is inhibited as the percent acetonitrile in the eluent is increased, thus lowering the detection efficiency of PAD. Peak area *versus* the percent acetonitrile in the eluent was tested in PAD using amygdalin as a representative cyanogenic glycoside. As the percent acetonitrile in the eluent was increased, the peak area of amygdalin was unaffected up to 15% (v/v) acetonitrile. At percent acetonitrile ≥ 18% (v/v) in the eluent, peak area was reduced (results not shown).

Detection limits were determined with both the 15 cm and 25 cm columns under gradient and isocratic elution. The limits of detection were determined at a signal-to-noise ratio of ca. 3. It should be noted that with PAD, as the percent acetonitrile in the eluent was increased, it was necessary to decrease the setting of sensitivity (i.e., increase attenuation) to reduce baseline noise. Table 2 shows the limits of detection found for the solutes using both UV and PAD detection methods based on a 20 μL injection. In gradient elution, using a 10 min linear gradient (from 0 to 60% acetonitrile) provided the lowest limit of detection for the solutes with PAD, and similar limits of detection were found for the 10 min and 20 min linear gradient with the UV detector. In gradient elution, PAD as well as UV detection required a lower setting for sensitivity (i.e., higher attenuation) in order to avoid a noisy baseline. An elution at 15% acetonitrile (v/v) provided the lowest limit of detection for isocratic analysis. The limit of detection in gradient elution was lower than in isocratic elution making gradient elution a more sensitive choice. In comparing UV and PAD, both detection methods provided similar results concerning the limits of detection for two solutes, namely amygdalin and prunasin. As expected, benzaldehyde, which is the degradation product of mandelonitrile, was detected at a lower level using UV detection than in PAD. This was the result of a weak response which is usually obtained for aldehydes using PAD.[15] The pulsed amperometric detector, however, was able to detect the presence of linamarin whereas UV detection did not; therefore, a combination of PAD and UV detection would provide the best method for the determination of the four solutes.

The linear dynamic range for the detector response was determined in both UV and PAD detection. The analytes amygdalin and prunasin were chromatographed isocratically with 15% (v/v) acetonitrile while linamarin was analyzed with a mobile phase at 6% (v/v) acetonitrile. Mandelonitrile was not tested for linear response due to its degradation to benzaldehyde. Using PAD, linamarin responded linearly in the concentration range of $1.25 \times 10^{-6} - 0.0075$ M ($R = 0.994$). For the analyte amygdalin and using UV detection the

Table 2

Limit of Detection (LOD) Obtained with UV and PAD in Terms of Molar Concentration

	LOD (mol/L) UV		LOD (mol/L) PAD	
Solute	**Isocratic**	**Gradient**	**Isoctatic**	**Gradient**
Linamarin	-----	-----	2.50×10^{-7}[a]	-----
	-----	-----	1.25×10^{-6}[b]	-----
	-----	-----	-----	1.25×10^{-6}[c]
	-----	-----	-----	2.50×10^{-7}[d]
	-----	-----	-----	9.83×10^{-6}[e]
Amygdalin	1.26×10^{-7}[f]	-----	7.58×10^{-7}[f]	-----
	4.40×10^{-7}[g]	-----	-----	-----
	-----	5.70×10^{-7}[h]	-----	1.00×10^{-6}[c]
	-----	6.83×10^{-7}[I]	-----	8.80×10^{-8}[d]
	-----	-----	-----	8.80×10^{-7}[e]
	1.00×10^{-7}[k]	-----	-----	-----
Prunasin	1.35×10^{-7}[f]	-----	1.35×10^{-6}[f]	-----
	4.68×10^{-7}[g]	-----	-----	-----
	-----	6.08×10^{-7}[h]	-----	1.35×10^{-6}[c]
	-----	7.28×10^{-7}[I]	-----	9.90×10^{-8}[d]
	-----	1.50×10^{-6}[j]	-----	9.90×10^{-6}[e]
	7.30×10^{-8}[k]	-----	-----	-----
Benzaldehyde	4.98×10^{-7}[g]	-----	1.25×10^{-5}[g]	----
	-----	1.53×10^{-7}[h]	-----	9.90×10^{-6}[c]
	-----	7.73×10^{-7}[I]	-----	9.90×10^{-7}[d]
	-----	1.50×10^{-6}[j]	-----	9.90×10^{-6}[e]

[a] Isocratic elution at 0% acetonitrile with a 15 cm column; [b] isocratic elution at 6% acetonitrile with a 15 cm column; [c] 20 min linear gradient with a 15 cm column; [d] 10 min linear gradient with a 15 cm column; [e] 6 min linear gradient with a 15 cm column; [f] isocratic elution at 12 % acetonitrile with a 15 cm column; [g] isocratic elution at 18% acetonitrile with a 15 cm column; [h] 20 min linear gradient with a 25 cm column; [I] 10 min linear gradient with a 25 cm column; [j] 6 min linear gradient with a 25 cm column; [k] isocratic elution at 15% acetonitrile with a 15 cm column. In [c, d, h and I] the gradient was from 0 to 60% acetonitrile while in [e and j], the gradient was from 18 to 60% acetonitrile.

response was linear (R = 0.995) in the concentration range of 1.00 x 10^{-7} – 0.007 M, while the detector response in PAD remained linear from 1.40 x 10^{-5} – 0.00138 M (R = 0.992). At 15% acetonitrile, the baseline noise with PAD was relatively high, reason for which the limit of detection is now about 1.40 x 10^{-5} M as opposed to 7.58 x 10^{-7} M at 12% acetonitrile in the eluent, see Table 2. In UV detection, prunasin maintained a range of linearity from 7.30 x 10^{-8} – 0.0080 M (R = 1.000), and in PAD the response was linear from 1.00 x 10^{-5} – 0.0083 M (R = 0.999). As with amygdalin, the baseline noise was relatively high at 15% acetonitrile permitting a limit of detection of only 1.00 x 10^{-5} M. In all cases, response was linear to the limits of detection for all of the analytes sampled.

In conclusion, the use of PAD and UV detection coupled with reversed phase chromatography provides a sensitive and efficient method for the determination and separation of cyanogenic glycosides. Under optimal conditions, linear detector response holds over a wide range of concentration for the solutes, and limits of detection are in the low micromolar to high nanomolar range (10^{-6} - 10^{-8} M).

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