Journal of Chromatography, 303 (1984) 77–88
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,951

CYANOGENIC GLYCOSIDES AND CYANOHYDRINS IN PLANT TISSUES

QUALITATIVE AND QUANTITATIVE DETERMINATION BY ENZYMATIC POST-COLUMN CLEAVAGE AND ELECTROCHEMICAL DETECTION, AFTER SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

LEON BRIMER* and LARS DALGAARD
Department of Pharmacognosy and Botany K, and Department of Chemistry BC, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø (Denmark)
(Received May 28th, 1984)

SUMMARY

A rapid, simple and reproducible high-performance liquid chromatography procedure is described for the qualitative and quantitative analysis of mixtures of cyanogenic glycosides.

The separation is achieved by means of a reversed-phase (C_8) column eluted with a phosphate buffer, pH 5.0, containing either 15 or 7.5% (v/v) methanol, 7.5% being necessary for resolution of epimeric pairs of the more hydrophilic glycosides. When this separation is combined with enzymatic post-column cleavage and electrochemical detection of the cyanide formed, a highly specific and very sensitive system is obtained.

The method was applied to cyanogenic glycosides in crude plant tissue extracts, and compared with both a thin-layer chromatographic method and to a traditional determination of total cyanide released after hydrolysis. Sensitivity, selectivity and accuracy were found sufficient to enable its routine use for analysis of food and fodder samples, for example. Cyanohydrins could be detected qualitatively.

INTRODUCTION

Cyanogenic glycosides, cyanogenic lipids and cyanohydrins are widely distributed in the plant kingdom, and the occurrence of cyanogenic glycosides in food and fodder is a particular problem in many parts of the world[1]. Thus, linamarin and to a lesser extent lotaustralin impart toxicity to *Manihot esculenta* Crantz (cassava), which in 1981 gave rise to an epidemic of tropical neuropathy in northern Mozambique[2,3].

Several procedures for the quantitative estimation of hydrogen cyanide (HCN, prussic acid) exist, but none can be regarded as reliable for the direct estimation of HCN released in plant homogenates. Thus, HCN must be liberated and isolated

0021-9673/84/$03.00

quantitatively from this matrix prior to estimation. Both of these operations, however, include several possible sources of errors[4]. Recently, one of the authors described a thin-layer chromatographic (TLC) densitometric method for the quantitative estimation of the genuine cyanogenic glycosides in extracts[5]. Unfortunately, this method is unable to distinguish between epimers, and may furthermore be inconvenient in the handling of a large number of analyses. Other methods for the determination of genuine cyanogenic compounds are few and unspecific, *e.g.* high-performance liquid chromatography (HPLC) monitored by a refractive index detector (for a discussion see ref. 6).

The separation of epimeric pairs, either for identification of an unknown cyanogenic compound or to establish the proportion between two epimers in a mixture, has always caused trouble when cyanogenic glycosides are concerned. Thus, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy has become widely accepted as standard for distinguishing *R* and *S* forms[7,8]. However, this calls for pure samples, so in recent years HPLC has been examined especially for its ability to resolve these forms. Although some resolutions have been achieved in a few cases[8], the results have been limited, and no separation system has proved universal.

Stationary phases in use for HPLC separation of cyanogenic glycosides include silica (straight phase) and octadecyl (RP-18) modified silica (reversed phase). With regard to the reversed-phase systems, the most common modifiers of the aqueous mobile phase are methanol, acetonitrile and 2-propanol, although acids such as acetic acid may be added when dealing with acidic glycosides, *e.g.* triglochinin[8].

Very recently post-column cleavage followed by electrochemical detection (ED) of glycosides (*e.g.* glucuronides) of different phenolic substances, separated by HPLC, has been demonstrated by Dalgaard *et al.*[9], as has a similar procedure for detection of cyanogenic glycosides[6,9]. The present paper deals with the development of an HPLC method for the separation —including epimeric pairs— and quantitative determination of cyanogenic glycosides (and cyanohydrins) in plant extracts, employing this latter method for detection.

EXPERIMENTAL

Instrumentation

The chromatographic system for selective detection consisted of an LDC Constametric Model III pump, equipped with a Rheodyne 7125 valve, a 250 × 4.6 mm I.D. Spherisorb RP-8, 5 μm (Phase Separations, U.K.) analytical column, a 50 × 3 mm I.D. enzymes reactor with Swagelock end fittings, a Kontron Uvikon LCD 725 UV detector 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 (potential 0.0 V). Numbers of theoretical plates for the analytical column in different systems were determined using a system consisting of a Kontron analytical LC 410 pump and an RI detector (Melz LCD 201, Berlin, F.R.G.). Further technical specifications and sketches of the chromatographic system for selective detection may be found in the refs. 6 and 9.

Enzyme reactors

The enzyme reactors were identical with reactor no. 2 described in ref. 6; descriptions of enzyme purification, coupling procedures, and activity measurements can also be found there.

Cyanogenic glycosides

Amygdalin (puriss. 419500, Fluka AG, Switzerland), linamarin (435971, Calbiochem, U.S.A.), prunasin (2-8929, Carl Roth, F.R.G.), dhurrin (a gift from Professor E. E. Conn), gynocardin and lucumin were available in our laboratories from previous work by Dr. R. Eyjolfsson. Proacacipetalin and proacaciberin were isolated from pods of *Acacia sieberana* var. *woodii* (Burtt Davy) Keay and Brenan as previously described for proacaciberin[10]. Sambunigrin and holocalin were partly purified from extracts of *Sambucus nigra* L. (see below for details). Taxiphyllin was obtained as a crude sample from concentrated ethanolic extracts of *Taxus baccata* L. (♂) by a one-step purification [preparative TLC on silica gel on precoated plates (Merck 5747), with acetone–chloroform–water (85:10:5)]; the compound was visualized as described by Brimer *et al.*[5]. Heterodendrin and *epi*-heterodendrin were prepared from proacacipetalin and acacipetalin, respectively, by reduction as described elsewhere[5,11]. 3-Hydroxyheterodendrin was synthesized from proacacipetalin by the method of Brimer *et al.*[12]. The structures of cyanogenic glycosides are shown in Fig. 1.

α-Hydroxynitriles (cyanohydrins)

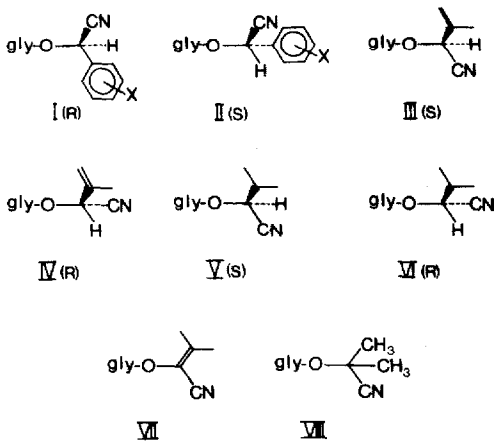
Cyanohydrins were obtained from parent glycosides by enzymatic cleavage (in Pi buffer, pH 5.0) of the glycosidic bond and inhibition, through addition of benzyl alcohol[13], of any hydroxynitrilase (cyanohydrinlyase) that might be present. For purification, the cyanohydrin can be partitioned between the aqueous solution and methylene chlorid (cyanohydrin in the lower phase). TLC was used to determine the time needed for total hydrolysis[5]. As the source of glycosidases, β-glucuronidase preparation *Helix pomatia* (Sigma G-0876) was used.

Epimerization and isomerization of cyanogenic glycosides

The glycosides amygdalin (*R*), prunasin (*R*), sambunigrin (*S*), holocalin (*R*), and lucumin (*R*) were epimerized to give mixtures of the *R* and the *S* epimers (amygdalin–neoamygdalin, prunasin–sambunigrin, holocalin–zierin and lucumin–epilucumin) by treatment with an 0.01 *N* aqueous solution of ammonia[14]. Proacacipetalin and proacaciberin were epimerized by treatment with 0.7% triethylamine in water for 15 min and isomerized to acacipetalin and acaciberin respectively with 15% triethylamine for 3 h[15].

Preparation of extracts for HPLC analysis

Extracts were prepared from leaves of *Sambucus nigra* L. collected on May 25th, 1983, at a roadside in Lyngby north of Copenhagen, from leaves of *Prunus lauracerasus* L. c.v. *Schripkaensis* collected on May 25th, 1983, in Botanical Garden, University of Copenhagen (plant no. 1974/5112) and from neadels of *Taxus baccata* ♂ L. collected the same date and place (plant no. 1816 B/1). *P. lauracerasus* and *T. baccata* are well known to contain prunasin and taxiphyllin, respectively.



Glucoside	*Structural formula*	*gly*	*X*
1 Prunasin	I	β-D-Glucosyl	H
2 Amygdalin	I	β-Gentiobiosyl	H
3 Lucumin	I	β-Primeverosyl	H
4 Taxiphyllin	I	β-D-Glucosyl	*p*-OH
5 Holocalin	I	β-D-Glucosyl	*o*-OH
6 Sambunigrin	II	β-D-Glucosyl	H
7 Neoamygdalin	II	β-Gentiobiosyl	H
8 Epilucumin	II	β-Primeverosyl	H
9 Dhurrin	II	β-D-Glucosyl	*p*-OH
10 Zierin	II	β-D-Glucosyl	*o*-OH
11 Proacacipetalin	III	β-D-Glucosyl	–
12 Proacaciberin	III	β-Vicianosyl	–
13 Epiproacacipetalin	IV	β-D-Glucosyl	–
14 Epiproacaciberin	IV	β-Vicianosyl	–
15 Heterodendrin	V	β-D-Glucosyl	–
16 Epiheterodendrin	VI	β-D-Glucosyl	–
17 Acacipetalin	VII	β-D-Glucosyl	–
18 Acaciberin	VII	β-Vicianosyl	–
19 Linamarin	VIII	β-D-Glucosyl	–

Fig. 1. Structural formulae of the cyanogenic glycosides studied (*cf.* Table II).

Plant materials were air-dried for 48 h at 40°C, and 1 part (by weight) of material was soaked with 20 volume parts of 70–80% (v/v) boiling methanol. The mixture was treated in a blender (Waring, one quart) at 21,000 rpm. The mixture was decanted, the procedure repeated twice, and the extracts were combined and made up to 60 parts by volume. This crude extract was concentrated *in vacuo* at a bath temperature below 40°C to 10 parts by volume, and left overnight for precipitation at 5°C. The supernatant was evaporated *in vacuo* and dissolved in 15% methanol to make 2.5 parts by volume (concentrated extracts).

Purification and identification of cyanogenic compounds present in the extract from S. nigra

Until recently, only sambunigrin was identified as a cyanogenic constituent in *S. nigra* L. (common elder). However, in 1973 Jensen and Nielsen[16] showed that prunasin, zierin and holocalin were also present in some samples from the isle of Sjælland (Denmark). Thus it was found necessary to characterize the extract used for analysis with respect to cyanogenic constituents in order to interpret the chromatographic results. For this purpose, the two main cyanogenic constituents were partly purified from extracts. After identification, these purified compounds further served as samples for epimerization experiments.

Purification

Concentrated extracts (see above) were evaporated *in vacuo* and further purified by a two-step partition, first in methanol–chloroform–water (300:125:150; glycosides in top phase), then in water–2-propanol (glycoside in top phase), followed by two steps of column chromatography, first on silica gel with ethyl acetate–methylbenzene–ethanol (4:1:1) then on silica gel with acetone–chloroform (9:1). This yielded two crude samples containing different cyanogenic compounds, shown to be sambunigrin and holocalin, respectively (see below). Final purification of each compound was achieved by reversed-phase (RP-8) preparative HPLC on a LiChrosorb RP-8 7 μm column (250 × 16 mm I.D.), with a mobile phase of methanol–water (15:85) at a flow-rate of 6.5 ml/min.

Identification

Sambunigrin. The ^{13}C NMR spectrum in deuterium oxide [methanol (δ 49.7) as internal standard] at 67.9 MHz proved to be identical with that reported by Hübel *et al.*[17] and slightly different from that of authentic prunasin. The compound chromatographed together with sambunigrin on TLC [silica gel, ethyl acetate–acetone – chloroform–methanol–water (40:30:12:10:8)][5] and gave a brown colour (different from the blue-gray produced by prunasin) when sprayed with hydroxylamine–iron-(III) chloride after the separation on TLC[18,19]. The compound had identical chromatographic properties with those of sambunigrin (produced from prunasin by epimerization, see above) on HPLC, and afforded a mixture of two compounds chromatographing together with sambunigrin and prunasin, respectively, when treated as described under epimerization.

Holocalin. The ^{13}C NMR spectrum in deuterium oxide proved to be identical with the reported spectrum[17]. The compound chromatographed with the holocalin–zierin mixture on TLC[5] and gave a blue-gray colour with hydroxylamine–iron(III) chloride (different from the brown colour produced by dhurrin). The compound had chromatographic properties different from these of dhurrin and taxiphyllin on HPLC, and afforded a mixture showing two peaks (one identical with the original) when treated as described under epimerization. The order of elution from the column was as expected for reversed-phase systems[8].

Quantitative analysis of cyanogenic compounds present in extracts prepared for HPLC analysis

Extracts were investigated on straight-phase TLC employing specific quanti-

TABLE I

DETERMINATION OF CYANOGENIC GLYCOSIDES, AND TOTAL HNC RELEASED, FROM EXTRACTS OF *SAMBUCUS NIGRA* L., *PRUNUS LAURACERACUS* L. AND *TAXUS BACCATA* L.

$k'$is = k' for added internal standard, *i.e.* amygdalin.

Extract	*Compound(s)*	*HPLC [specific detection (ED)]***			*Compounds****	TLC***		*Hydrolysis in solution total HCN*
		k'	$k'/k'is$	*μmol/ml F = ± S.D.*		"R_F"	*μmol/ml ± S.D.*	*(μmol/ml ± S.D.)*
S. nigra (d.f. 50)*	Holocalin	3.2	0.64	1.9 ± 0.3 (n=3)	Holocalin–zierin	0.62	1.4 ± 0.2 (n=4)	6.9 ± 0.4 (n=4)
	Prunasin	6.9	1.39	0.2 ± 0.1 (n=3)	Prunasin–sambunigrin	0.67	4.6 ± 0.4 (n=4)	
	Sambunigrin	7.3	1.47	3.7 ± 0.3 (n=3)				
S. nigra (d.f. 500, s.f)*	Holocalin	3.6	0.69	1.8 ± 0.1 (n=3)				
	Prunasin	7.1	1.34	0.34 ± 0.02 (n=3)				
	Sambunigrin	7.6	1.42	3.8 ± 0.1 (n=3)				
P. lauracerasus (d.f. 500)*	Prunasin	6.8	1.37	19.3 ± 1.1 (n=3)	Prunasin–sambunigrin	0.67	17.4 ± 1.2 (n=4)	20.3 ± 1.0 (n=4)
T. baccata (d.f. 50)*	Taxiphyllin	2.0	0.42	0.6 (n=1)	Taxiphyllin dhurrin	0.64	0.78 ± 0.1 (n=4)	0.71 ± 0.02 (n=4)
T. baccata (d.f. 50, s.f)*	Taxiphyllin	2.0	0.42	0.6 (n=1)				
Glycosides								
Dhurrin		2.1	0.40					
Holocalin–zierin		3.1	0.62					
Amygdalin		5.0	1.0					

* d.f. = dilution factor. Analysis of "concentrated extracts" (see under Experimental) was performed after proper dilution, the detection limit of the method being in the low picomole range (ref. 6). Injection volume 20 μl; s.f. = split flow, 25% through enzyme reactor for detection (corresponding to flow-rate through reactor of 0.25 ml/min).

** Some of these separations monitored specific (post-column cleavage/ED), by RI or UV are shown in Figs. 2–4. Column and other conditions as in text. Eluent: phosphate buffer–methanol (85:15); flow-rate, 1 ml/min. Post-column reaction conditions for ED: reagent 2 *M* sodium hydroxide; reagent flow-rate, 0.2 ml/min; detector sensitivity, 0.05 μA or 0.5 μA.

*** The TLC method is unable to distinguish between the two epimeric forms. "R_F": the chromatograms were run twice (2 ascents), and "R_F" calculated as the distance to compound divided by the distance to solvent front.

tative detection as described by Brimer *et al.*[5]. The analytical system was: Sigel (precoated plates, Merck), with ethyl acetate–acetone–chloroform–methanol–water (40:30:12:10:8, 2 ascents). Extracts were further analyzed for total cyanogenic capacity by enzymatic hydrolysis of the cyanogenic compounds in aqueous solution, and colorimetric determination of released cyanide (after Epstein, as modified by Jørgensen[20]). For further details, see ref. 5. Results are presented in Table I, together with the HPLC based determinations.

RESULTS AND DISCUSSION

Separation of glycosides

In the present work, separations were performed by means of reversed-phase systems based on octyl (RP-8) modified silica as stationary phase, with methanol–water or methanol–phosphate buffer mixtures as eluent. Restricting the experiments to isocratic elution, it was found that acceptable results could be obtained with concentrations of modifier (methanol) below or equal to 15% (v/v). This permitted use of the highly specific detection system[6,9], consisting of post-column cleavage and ED with silver electrode, with buffered solvents in order to optimize the enzymatic cleavage and protect the enzyme reactor.

Table II lists the cyanogenic glycosides and their chromatographic behaviour in the different systems. Note that the k' values in water–methanol mixtures differ slightly from those found in buffered eluent, even though the compounds mentioned have no proteolytic groups. This phenomenon has been observed previously, however.

The data in Table II clearly demonstrate that good resolution of epimeric pairs can be obtained, simultaneously with a useful separation of glycosides with different aglycone and/or sugar parts. Thus, the glycosides with non-hydroxylated aromatic aglycones could be analysed by means of the 15% methanol system, whereas the remaining compounds require a 7.5% system if epimeric resolution is to be obtained.

Detection of separated glycosides

In order to examine the combination of these separation systems with the detection system mentioned earlier, different extracts of cyanogenic plants were analysed. Extracts were prepared essentially as described earlier[5], a method shown to provide quantitative extraction of several cyanogenic glycosides; the fate of cyanohydrins and free cyanide during this procedure was not dealt with. The results were compared with conventional analysis for released total cyanide, and with a TLC analysis of the same extracts. The results are listed in Table I. On the one hand, they illustrate the severe problems faced in the analysis of cyanogenic compounds when dealing with crude extracts, as the total cyanide found differs to quite an extent. On the other hand, it is clear that the HPLC method examined provides quantitative results comparable with those obtained by other methods, and with at least the same degree of precision. Together with this, qualitative data for identification down to the level of configuration at C-2 (epimeric resolution) are easily obtained, by means of the absolute (k') or the relative (to internal standards) chromatographic properties of the compounds.

In this work, the quantification of the cyanogenic compounds is based on

TABLE II

CHROMATOGRAPHIC PARAMETERS, SEPARATION CHARACTERISTICS OF EPIMERS AND DOUBLE BOND ISOMERS UNDER DIFFERENT CONDITIONS; SOME TYPICAL EXAMPLES

k' = capacity factor ($k' = \frac{V_r - V_v}{V_v} = \frac{V_r'}{V_v}$), V_r = retention volume for solute, V_v = void volume (H_2O peak detected by RI = 2.4 ml). R_s = resolution factor $\left\{R_s = 2\frac{[V_r(1) - V_r(2)]}{W(1) + W(2)}\right\}$, *i.e.* difference between retention volumes, divided by the average band width.

Number of theoretical plates $N\left[N = 5.54 \times \left(\frac{V_r'}{W_{\frac{1}{2}}}\right)^2\right]$ for the column, *ca.* 5000 as calculated for amygdalin and prunasin in water–methanol (85:15).

*Solute**	*Configuration at C-2*	*Eluent*					
		Water–methanol (92.5:7.5)		*Water–methanol (85:15)*		*Phosphate buffer**–methanol (85:15)*	
		k'	R_s	k'	R_s	k'	R_s
Linamarin (19)	–	2.1		0.7			
Epiproacacipetalin (13)	*R*	5.2	1.0				
Proacacipetalin (11)	*S*	5.6				3.3	
Acacipetalin (17)	–	8.2					
Taxiphyllin (4)	*R*	6.2	0.9	1.9	small		
Dhurrin (9)	*S*	6.6		2.0			
Epiheterodendrin (16)	*R*	6.5	1.3				
Heterodendrin (15)	*S*	7.4				3.4	
Epiproacaciberin (14)	*R*	8.6	0.8				
Proacaciberin (9)	*S*	9.1		2.2			
Acaciberin (18)	–	13.5					
Holocalin (5)	*R*	9.1		3.0	small		
Zierin (10)	*S*	9.6		3.1			
Neoamygdalin (7)	*S*	17.0	1.3				
Amygdalin (2)	*R*	18.4		4.5		5.0	
Prunasin (1)	*R*	17.1	1.1	5.9	0.9	6.6	0.9
Sambunigrin (6)	*S*	18.5		6.4		7.1	
Lucumin (3)	*R*	28.4	1.4	6.8		7.5	0.7
Epilucumin (8)	*S*	31.1				7.9	

* Structure number from Fig. 1. in paranthesis.

** Phosphate buffer, 0.05 *M* potassium phosphate buffer pH 5.0. No correction for pH changes was made when methanol was added.

comparison with an added internal standard, namely amygdalin. As the ultimate cleavage product (CN^-) obtained through the addition of sodium hydroxide after the enzyme reactor but prior to detection, is the same for all the cyanogenic compounds, quantification is possible by comparing the peak areas if (and only if) the compounds have been totally hydrolysed in the enzyme reactor[6]. The degree of hydrolysis is a function of reactor activity, substrate affinity, temperature, pH, solvent composition and the residence time of substrate in the reactor[6,9]. In the present study, the enzyme reactor contained partly purified glycosidases from *Helix pomatia* (the wineyard snail), and afforded total hydrolysis of the glycosides present in the extracts

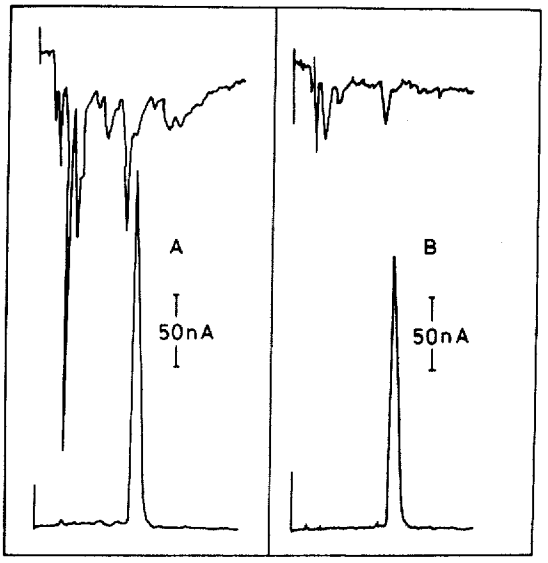


Fig. 2. Series dual detector chromatograms of extracts from leaves of *P. lauracerasus* L. (A) Crude extract, diluted eleven times with mobile phase; (B) concentrated extract, diluted 500 times with mobile phase. Mobile phases, 15% (v/v) methanol in 0.05 *M* phosphate buffer (pH 5.0); flow-rate, 1.0 ml/min. Detection: upper track, UV (upstream, wavelength 254 nm); lower track, ED (downstream). Other conditions as in Table I.

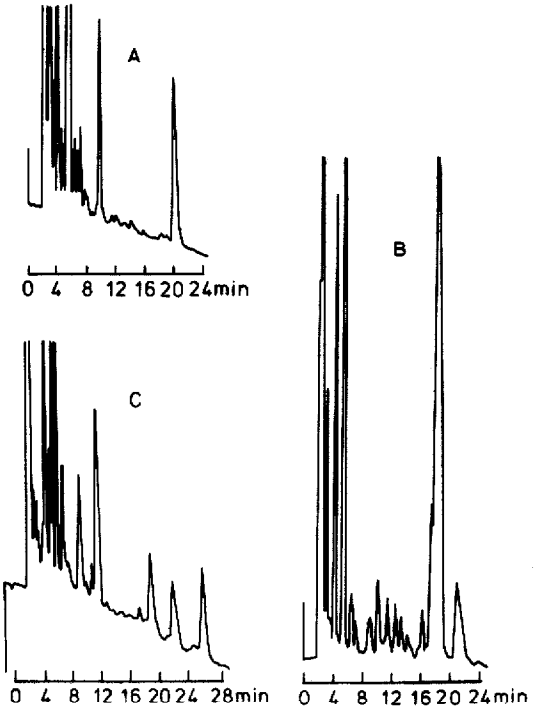


Fig. 3. Chromatograms of different plant extracts. (A) Concentrated extract of *S. nigra* L.; (B) concentrated extract of *P. lauracerasus* L.; (C) concentrated extract of *T. baccata* L. Detection, refraction index (RI). Conditions as in Table I.

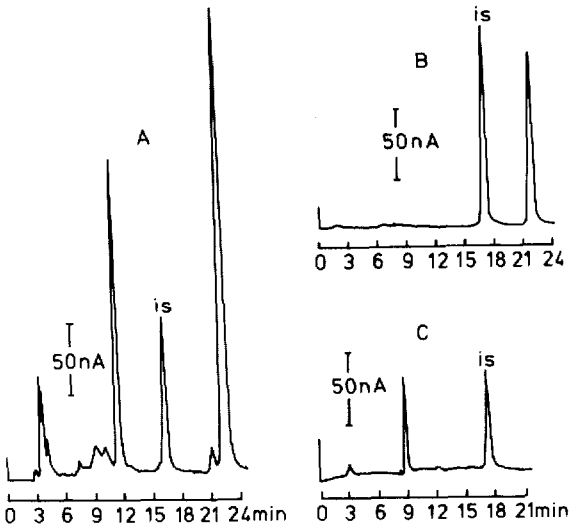


Fig. 4. Chromatograms of the same plant extracts as shown in Fig. 3, after suitable dilution. (A) *S. nigra* L. (d.f. 50); (B) *P. lauracerasus* L.; (C) *T. baccata* L. (d.f. 50). Amygdalin added as internal standard (is). Other conditions as in Fig. 3 and Table I. For identification of peaks, see Table I.

and of the internal standard, under the conditions used. If total hydrolysis cannot be obtained for one or more compounds, the split-flow technique may be used to increase the residence time and hence the degree of cleavage. Results using this technique is reported for both *S. nigra* and *T. baccata* extracts in Table I. The construction and theoretical aspects of split flow, the limit of detection, and the practical control of whether total hydrolysis has been obtained or not, are discussed in ref. 6. Analysis of single low affinity (*e.g.* linamarin) compounds based on standard curves, under conditions with only partial hydrolysis, are dealt with in the same paper.

The advantages of the specific detection used can be illustrated by comparison of chromatograms of the same extracts when monitored by RI or UV. As can be seen in Fig. 2, UV absorption at 254 nm does not allow detection, whether crude or "concentrated" extracts are used. Greater specificity can be obtained when changing the wavelength to the optima for the different glycosides[21], although this renders the method less versatile. Moreover, UV detection is fruitful only for glycosides with a conjugated double bond system (*e.g.* triglochinin) or aromatic moieties in the aglycone. RI detection is a possibility, as pointed out by Nahrstedt[21], especially when dealing with higher concentrations of the cyanogenic constituent(s) in question. Fig. 3 illustrates this, but also shows that detection is far from specific, and that baseline problems often arise even in the case of partly purified samples such as the "concentrated" extracts used here. Both UV and RI detection demand a standard curve or a response factor for each compound. In comparison, the chromatograms obtained when using post-column cleavage and ED make identification as well as quantification simple (Table I, Figs. 2 and 4). The concentrated extracts analysed contained no green pigments, and were prepared in such a way as to protect the column. However, the crude extracts could be analysed too, as shown in Fig. 2.

Cyanohydrins

Cyanohydrins (α-hydroxynitriles) may be present in material of biological origin either as natural constituents[7,11] or as a result of hydrolytic decomposition of cyanogenic glycosides or lipids. This latter situation may be seen in poorly stored or intentionally fermented material, such as fermented cassava (*Manihot esculenta* Crantz) products.

The detection of cyanohydrins predictably proved to be independent of the presence of an enzyme reactor. This enables ready discrimination from the cyanogenic glycosides. Quantitative determination, however, requires that the compounds are stable during sample preparation, injection and chromatographic separation. Because cyanohydrins are generally unstable, the stability in aqueous solution being highly dependent on the pH *inter alia*[22], this must be ensured for each compound through experiments under different conditions.

In this work, it was demonstrated that all the cyanohydrins selected, *i.e.* 2-hydroxy-3-methyl-3-butenenitrile, 2-hydroxy-3-methylbutanenitrile, 2,3-dihydroxy-3-methylbutanenitrile and 2-hydroxy-2-phenylethanenitrile (mandelonitrile), could be detected after separation on the reversed-phase column (Fig. 5). These cyanohydrins correspond to (are aglycones from, among others) the cyanogenic glucosides proacacipetalin, heterodendrin, 3-hydroxyheterodendrin and prunasin, respectively, from which they were prepared.

In spite of the instability of cyanohydrins, successful quantitative determination of mandelonitrile has been achieved, even by use of a TLC–densitometric method[9].

CONCLUSION

HPLC on a reversed-phase (RP-8) stationary phase and enzymatic post-column cleavage combined with ED appears to be a practical method for detection of

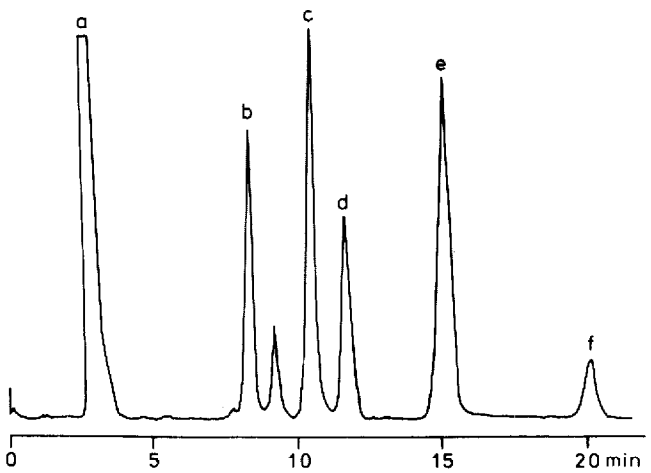


Fig. 5. Chromatographic separation of different glycosides and a cyanohydrin corresponding to one of the glycosides. Peaks: a = benzyl alcohol (saturated solution); b = proacacipetalin; c = heterodendrin; d = 2-hydroxy-3-methylbutanenitrile (corresponding to proacacipetalin); e = amygdalin; f = prunasin. Mobile phases, 15% (v/v) methanol in phosphate buffer 0.05 *M* (pH 5.0). Other conditions as in Table I. Electrochemical detection.

cyanogenic constituents in extracts and biological fluids. It is particularly suitable for the quantitative determination of epimeric mixtures, and the results demonstrate that HPLC will be able to provide this determination in a reliable manner. Investigations aimed at establishing HPLC methods for the quantitative determination of cyanide and all cyanogenic compounds (including cyanohydrins and groups of glycosides not discussed in this paper) that might be present in a product would be valuable.

Of the sources for hydrolytic enzymes (glycosidases) commercially available at present, only the β-glucuronidase preparation *Helix pomatia* contains activity towards such widely different cyanogenic glycosides as linamarin and amygdalin, for example. Further work, with a view to finding new and cheaper sources for enzymes, are in progress. Thus, crude enzyme preparations from the fungus *Stemphylium loti* Graham (which causes leafspot on *Lotus corniculatus* L.) were shown to possess activity against both amygdalin and linamarin[23]. These results have been confirmed in our laboratory, where activity towards gynocardin was also established.

ACKNOWLEDGEMENTS

We thank Professor E. E. Conn, University of California, for the sample of dhurrin, and Dr. K. Schaumburg (Copenhagen University) for recording the NMR spectra with the Bruker HX 270 S spectrometer of the Danish Natural Science Research Council.

REFERENCES

1 F. Nartey, in B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing (Editors), *Cyanide in Biology*, Academic Press, New York, 1981, p. 115.
2 J. Cliff, A. Martelli, A. Molin and H. Rosling, *Mantakassa: An Epidemic of Spastic Paraparesis Associated with Chronic Cyanide Intoxication in a Cassava staple Area of Mozambique. Part I: Epidemiological and Clinical and Laboratory Findings in Patients*, WHO bulletin 62, 1984, p. 477.
3 E. Casadei, P. Jansen, A. Molin, A. Rodrigues and H. Rosling, *Mantakassa: An Epidemic of Spastic Paraparesis Associated with Chronic Cyanide Intoxication in a Cassava staple Area of Mozambique. Part II: Nutritional Factors and Hydrocyanic Acid Content of Cassava products*, WHO bulletin 62, 1984, p. 485.
4 A. Nahrstedt, W. Erb and H.-D. Zinsmeister, in B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing (Editors), *Cyanide in Biology*, Academic Press, New York, 1981, p. 461.
5 L. Brimer, S. B. Christensen, P. Mølgaard and F. Nartey, *J. Agr. Food Chem.*, 31 (1983) 789.
6 L. Dalgaard and L. Brimer, *J. Chromatogr.*, 303 (1984) 67.
7 D. S. Siegler, in L. Reinhold, J. G. Harborne and T. Swaine (Editors), *Progr. Phytochem.*, 4 (1977) 83.
8 A. Nahrstedt, in B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing (Editors), *Cyanide in Biology*, Academic Press, New York, 1981, p. 145.
9 L. Dalgaard, L. Nordholm and L. Brimer, *J. Chromatogr.*, 265 (1983) 183.
10 F. Nartey, L. Brimer and S. B. Christensen, *Phytochemistry*, 20 (1981) 1311.
11 L. Brimer, S. B. Christensen and F. Nartey, *Abstracts of the 14th International Symposium on the Chemistry of Natural Products, IUPAC, Poznan, Poland, 1984*, Vol. I, 1984, p. 227.
12 L. Brimer, S. B. Christensen, J. W. Jaroszewski and F. Nartey, *Phytochemistry*, 20 (1981) 2221.
13 M. S. Jorns, *Biochim. Biophys. Acta*, 613 (1980) 203.
14 A. Nahrstedt, *J. Chromatogr.*, 152 (1978) 265.
15 M. G. Ettlinger, J. W. Jaroszewski, S. R. Jensen, B. J. Nielsen and F. Nartey, *J. Chem. Soc., Chem. Commun.*, (1977) 952.
16 S. R. Jensen and B. J. Nielsen, *Acta Chem. Scand.*, 27 (1973) 2661.
17 W. Hübel, A. Nahrstedt and V. Wray, *Arch. Pharm.*, 314 (1981) 609.
18 U. Schwarzmaier, *J. Chromatogr.*, 111 (1975) 430.
19 F. van Valen, *Proc. Koninkl. Akad. Wetensch.*, 81C, (1978) 132.
20 K. Jørgensen, *Acta Chem. Scand.*, 9 (1955) 548.
21 A. Nahrstedt, *J. Chromatogr.*, 177 (1979) 157.
22 R. D. Cooke, *J. Sci. Food. Agr.*, 29 (1978) 345.
23 R. L. Millar and R. Hemphill, *Physiol. Plant. Pathol.*, 13 (1978) 259.