the ratio of α-ergokryptine to β-ergokryptine was around 5 to 1. The other large signal observed in the sample extract was for m/z 294 daughters (22% as intense as m/z 342 daughters). The m/z 294 daughter spectrum had a m/z 195 ion as the base peak with a small peak (~2% of that of m/z 195) at m/z 209, indicating ergocornine was the major peptide alkaloid shown by the m/z 294 daughters from the sclerotia extract matrix. Because the daughter signals for the ergoptine and β-ergoptine were overshadowed by daughter signals from ergocornine (50 to 1 concentration ratio), the presence of ergoptine isomers was noted; however, the determination of which ones were present could not be made.

Analysis of the small signal observed for m/z 280 daughters from the sample (~3% as intense as for m/z 342 daughters) revealed the presence of ergosine isomers and ergoine. The base peak in the m/z 280 daughters was at m/z 209 (100), with smaller peaks observed at m/z 195 (7) and m/z 181 (3). The m/z 209 base peak in the m/z 208 daughter spectrum indicated that ergosine and/or β-ergosine were present in the matrix. Both compounds probably were present, as indicated by daughter signals at m/z 237 (ergosine) and at m/z 265 and m/z 259 (β-ergosine). The signal at m/z 195 indicated the presence of a smaller amount of ergonine, whereas the small signal at m/z 181 suggested ergobutyrine, although no standard for ergobutyrine was available. A small signal was observed for m/z 266 daughters (~1% as intense as the m/z 342 daughter signal). This signal was clearly observable above the system noise; the m/z 266 daughter spectrum from the barley matrix was identical with the m/z 266 daughter spectrum for ergovaline, indicating its presence in the sample. Thus, the presence of at least 10 of the 14 peptide alkaloids could be demonstrated by MS/MS in a crude extract of a sample suspected to contain ergot alkaloids. MS/MS in a single analysis has rapidly identified the peptide alkaloids from a simple extract. The detection and identification of these compounds by MS/MS were on approximately 1 μg of crude alkaloid material (colorimetrically determined by using p-(dimethylamino)benzaldehyde) and amply demonstrates the applicability of this technique for the analyses of the individual ergot peptide alkoids without the rigorous cleanup required by current procedures.

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Determination of Cyanogenic Compounds by Thin-Layer Chromatography. 1. A Densitometric Method for Quantification of Cyanogenic Glycosides, Employing Enzyme Preparations (β-Glucuronidase) from *Helix pomatia* and Picrate-Impregnated Ion-Exchange Sheets

Leon Brimer,* Søren Brøgger Christensen, Per Mølgaard, and Frederick Nartey

A densitometric method for the quantitative determination of cyanogenic glycosides is described. The method is based on the release of HCN catalyzed by the enzyme preparation β-glucuronidase from *Helix pomatia* and subsequent direct detection of HCN on hydrophobic, picrate-impregnated, transparent, ion-exchange sheets. The sheets are placed directly on the enzyme-wetted chromatogram, and the intensities of the obtained spots are determined. No significant changes in intensities of spots occur over a period of 28 days, if the sheets are protected from corrosive vapors. If a densitometer is not available, or when a rapid field test is required, a semiquantitative determination is possible by visual inspection. The method was found suitable for the separate estimation of cyanogenic principles in cassava meal, lima beans, and linseed meal.

The presence of cyanogenic constituents, i.e., glycosides, cyanogenic lipids, and cyanhydrins in food and fodder of plant origin, is a severe problem in many parts of the developing countries. Especially the occurrence of considerable amounts of linamarin and lotaustralin in *Manihot esculenta* Crantz (cassava) is troublesome, since this plant and its products form one of the most important sources of dietary carbohydrate for millions of people (Phillips, 1977; Nartey, 1978). Similarly, *Phaseolus lunatus* L. (lima bean), an important edible legume especially grown in South and Latin America, contains linamarin and lotaustralin (Conn, 1979), while the major cyanogenic constituents in the widely used fodder product linseed

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meal (from *Linum usitatissimum* L.) recently has been characterized as linustatin and neolinustatin (Smith et al., 1980). In spite of this, no simple, rapid, and inexpensive technique is available for the quantification of single resolved cyanogenic constituents in complex mixtures. Gas chromatographic and high-performance liquid chromatographic methods for analyses of cyanogenic glycosides have been reported (Nahrstedt, 1970, 1973, 1979). Both of these procedures, however, suffer from the requirement of expensive equipment and the lack of specificity in the detection procedure.

Since the introduction of the picric acid test for the detection of hydrogen cyanide by Guignard (1906), several other color reagents have been reported (Eyjolfsson, 1970; Carducci et al., 1972; Conn, 1980), none of these, however, being used as widely as the Guignard reaction. In 1960, Butler and Butler described a technique which made this test suitable for visualization of cyanogenic compounds on paper and thin-layer chromatograms. In this method the chromatogram is sprayed with an enzyme solution, a perforated sheet is placed on the moist chromatogram, and on top of this, a paper impregnated with a basic picrate solution is placed. After cleavage of the cyanogenic components, the released hydrogen cyanide reacts with the picrate to give orange brown spots. The disadvantages of this kind of method are the space between the chromatogram and the reagent paper, which results in diffuse spots, and the nontransparency of the paper, making densitometric determination difficult. Sandwich methods based on other hydrogen cyanide reagents have been reported (Bennett and Tapper, 1968; Tantisewie et al., 1969); none of these, however, are suitable for quantitative analysis. A further complication of the sandwich methods is the resistance of a number of cyanogenic glycosides, among these linamarin, lotaustralin, and gynocardin, toward the only commercially available and generally used β-glucosidase (almond). For this reason, several methods for the preparation of crude enzyme solutions (linamarases/gynocardases) from different sources have been presented (Nartey, 1968; Ikediobi et al., 1980; Cooke, 1981).

In order to establish a modification of the sandwich method for quantitative measurements, transparent and hydrophobic reagent sheets are needed; they can be placed in direct contact with the enzyme-wetted chromatogram, thus minimizing the diffusion of HCN and allowing densitometric absorbance measurements. Furthermore, an enzyme preparation that liberates hydrogen cyanide quantitatively from all cyanogenic compounds is required. We hereby report a modification of the sandwich method of Butler and Butler (1960), which enables selective visualization and quantification of the enzymatically released hydrogen cyanide, after separation of the constituents by thin-layer chromatography. Thin-layer systems for separation of the compounds in question may often be found in the literature [refer, for example, to Siegler (1977), Spencer et al. (1982), and Tjon Sie Fat (1979)]. In our laboratory, the following system has proved valuable: Si gel (precoated plates, Merck 5721), EtOAc–$(CH_3)_2CO$–$CHCl_3$–MeOH–H_2O (40:30:12:10:8); two ascents; R_f relative to amygdalin, prunasin/sambunigrin 2.3, dhurrin 2.2, proacacipetalin 2.2, holocalin 2.1, lotaustralin 1.9, linamarin 1.8, cardiospermin 1.7, 3-hydroxy-heterodenrin 1.7, gynocardin 1.5, lucumin 1.4, proacaciberin 1.1, cardiosperminsulfat 0.6, triglochinin 0.2.

The method described has been found to allow quantification of 16 cyanogenic glycosides, representing nearly all known types of aglycons (e.g., phenylalanine-, valine-, isoleucine-, and leucine-derived glycosides together with

cyclopentene glycosides) and all sugars known to occur in cyanogenic glycosides (glucose, visianose, gentiobiose, and primeverose). A type of aglycon not represented above is the dihydropyridone derivative present in acalyphin (Nahrstedt et al., 1982).

MATERIALS AND METHODS

Chemicals. Amygdalin (puriss. 419500; Fluka AG, Switzerland), linamarin (435971; Calbiochem), prunasin (2-8929; Carl Roth, GFR), holocalin (a gift from Dr. L. Melchior Larsen), dhurrin (a gift from Prof. E. E. Conn), triglochinin (a gift from Dr. J. Jaroszewski), and cardiospermin and cardiospermin sulfate (gifts from Prof. A. Nahrstedt and Dr. W. Hübel) were used. Heterodendrin was prepared from proacacipetalin by reduction (100 mg of proacacipetalin in 20 mL of EtOH, 100 mg of 10% Pd/C, H_2 pressure 50 bar, stirred 1 h at 23 °C), essentially the method of Hübel (1978). 3-Hydroxyheterodendrin was prepared as described earlier (Brimer et al., 1981). Lucumin and gynocardin were present in the department due to earlier work of Dr. R. Eyjolfsson; the remaining glycosides were isolated from natural sources by the authors. β-Glucuronidase from *Helix pomatia* [G-0876 (crude solution) and G-0751 (partially purified powder), Sigma Chemical Co.] and β-glucosidase from almond (G-8625, Sigma Chemical Co.) were used.

Food and Fodder Samples. Linseed meal (a gift from Dlg., Copenhagen, Denmark, in the form of linseed extraction pellets). White lima beans (obtained from W. Atlee Borpee Co., Warminster, PA, through Thorvald Petersens Handelsgartneri, Copenhagen, Denmark). Unfermented cassava root meal (a gift from the director of the Federal Bureau of Industrial Research, Oshodi, Nigeria).

Preparation of Reagent Sheets. Precoated ion-exchange sheets [Polygram ionex 25-SB-Ac (MN)] were impregnated by consecutive immersion in the following three solutions: (1) a saturated solution of picric acid in water, followed by air-drying (hot air may be used); (2) a 1 M aqueous sodium carbonate solution, followed by air-drying (hot air may be used); (3) a 2% (w/v) ethanolic 1-hexadecanol (cetyl alcohol, USP/NF) solution, followed by drying at room temperature. The sheets may be stored for months either cold (0–5 °C) or at room temperature (about 20–23 °C) if protected from light and corrosive vapors as aldehydes, ketones, and nitriles. No differences were observed if the sheets were stored dry or at a humidity of 100%.

Incubation and Densitometric Analysis. After development, the chromatograms were sprayed with enzyme solution, either 0.1% w/v β-glucosidase (almond) in H_2O (approximately 5–6 units/mL) or an aqueous solution (final concentration about 6000 Fishman N (F) units/mL, resulting pH approximately 5.5) of β-glucuronidase (*H. pomatia*), about 1 mL/25 cm^2 when Si gel, 0.2 mm (precoated plates, Merck 5721), was used. The reagent sheet was immediately placed on the top and incubated for 25 h at room temperature. The absorbance of the spots was measured by the flying spot transmission technique [Vitatron TLD 100; light source, tungsten; secondary filter, 540 nm; mode, –log; aperture, 2.0 mm (circular) or 2.5 × 0.1 mm (rectangular)]. No significant changes in absorbance were observed after storing the developed sheets for 28 days, protected from light and corrosive vapors at room temperature (Figure 1).

Technical Problems. The impregnation of reagent sheets are best performed by means of a slow horizontal movement of the sheet through the solution. For the impregnation with 1-hexadecanol, the time of immersion

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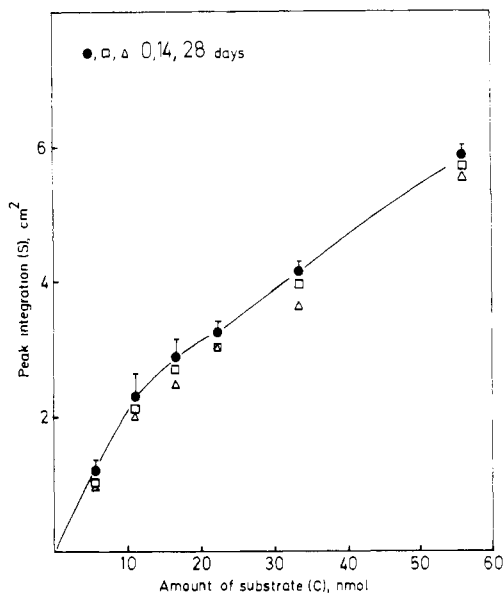
Figure 1. Calibration curves for amygdalin (β-glucosidase, n = 4) after 0, 14, and 28 days of storage (protected from light and corrosive vapors). Results given as mean ± SD.

must be limited; otherwise, the ion-exchange layer may loosen from the polyester sheet. Visual inspection of the prepared sheets for homogeneity proved to be a good quality control. Determination of calibration curves on different "impregnation batches" disclosed that small differences in sentivity may occur from batch to batch. It is thus essential always to determine a calibration curve on new batches of reagent sheets.

Preparation of Extracts of Food and Fodder. Over 1 part of material was poured 10 volume parts of 80% v/v boiling ethanol and the mixture was treated in a blender for 2 min at 21.000 rpm. After the mixture was decanted, the procedure was repeated twice, the combined extracts were filtered, and the filtrate was concentrated in vacuo at a bath temperature below 40 °C. Aliquots corresponding to 1 g of material were dissolved in 1-4 mL of 80% v/v ethanol. If the extraction of lima beans was performed in a flask under good stirring, only about 80% of the cyanogenic constituents were extracted. The remaining 20% was detected in the residue.

Colorimetric Determination of Total HCN Released. Materials, extracts, or glycosides corresponding to about 100 nmol of hydrogen cyanide were suspended in 2.0 mL of an aqueous enzyme solution (2000 F units/mL β-glucuronidase) and placed in the outer well of a conway microdiffusion cell. A sodium hydroxide solution (1.000 μL; 0.1 N) was placed in the center well, and the cell left for 48 h at 21-23 °C. The cyanide present in the sodium hydroxide solution was determined by the method of Epstein as modified by Jørgensen (1955). The colorimetric determination was performed in duplicate and the microdiffusion procedure repeated once or twice, using an enzyme solution containing 6000 F units/mL, in order to ensure quantitative hydrolysis. Results are given as mean ± standard deviation, the latter calculated as if the results were normally distributed.

RESULTS AND DISCUSSION

Irrespective of the use of β-glucosidase, glucuronidase, or a mixture of these enzyme preparations as spraying solutions, the calibration curves obtained for amygdalin, holocalin, and proacacipetalin were found not to differ significantly (Figure 2). Since β-glucosidase is known to cleave these glycosides quantitatively (Conn, 1980), this was concluded also to be true for glucuronidase under the
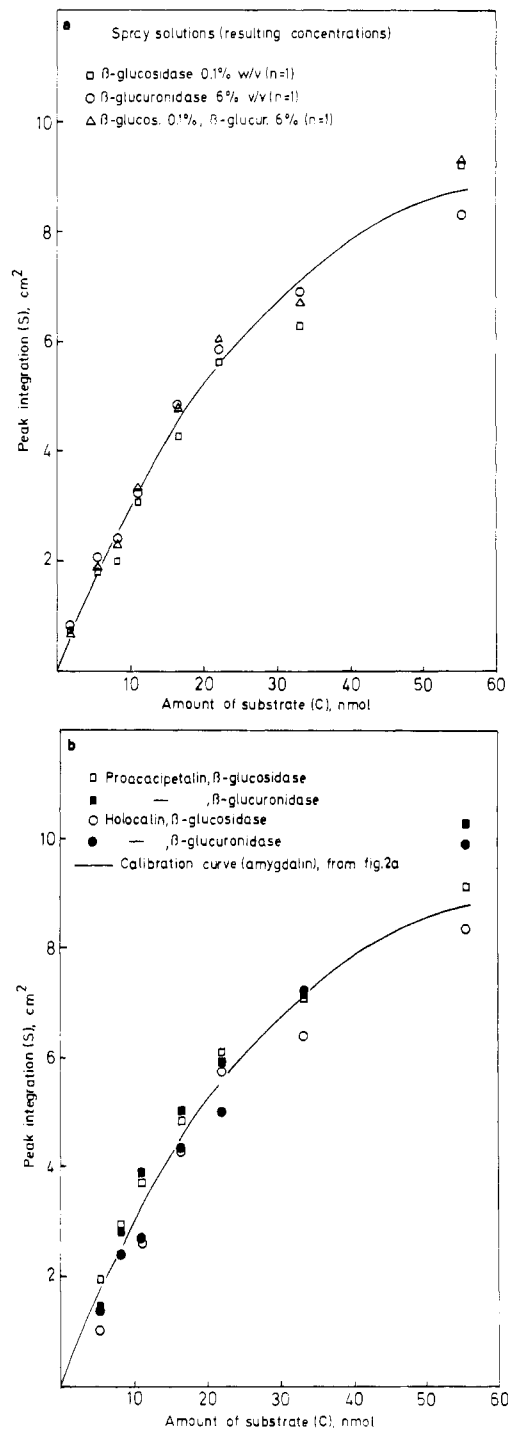
Figure 2. (a) Calibration curves for amygdalin as obtained with three different spray solutions. (b) Calibration curves for holocalin and proacacipetalin as obtained with two different spray solutions.

above mentioned conditions. Linearity of the calibration curves can be obtained mathematically up to approximately 70 nmol by the two step method of Bounias (1980), as illustrated below: A linear correlation (r = 0.992) between the natural logarithm of the signal response (area) and natural logarithm of the concentration of the cyanogenic compound with a slope 1.48 was demonstrated. By raising the signal response to the 1.48th power, we obtained linearity to the concentration of the cyanogenic constituent (r = 0.997).

In contrast to β-glucosidase, β-glucuronidase (*H. pomatia*) was found to give superimposable standard curves for 15 out of 16 glycosides tested, including linamarin, lotaustralin, gynocardin, and other glycosides only poorly cleaved by β-glucosidase (almond). Only 3-hydroxy-

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Table I. Degree of Cleavage of Cyanogenic Glycosides, As Determined by the TLC–Densitometric Method with Different Spray Solutions[a]

compound	solution		
	I	II	III
(R)-prunasin	q	q	q
(S)-sambunigrin	q	q	q
(R)-amygdalin	q	q	q
(R)-holocalin	q	q	q
(S)-dhurrin	q	q	q
triglochinin	q	q	q
(S)-proacacipetalin	q	q	q
(S)-heterodendrin	q	q	q
(S)-cardiospermin	q	q	q
(S)-cardiospermin sulfate	q	q	q
(R)-lucumin	p	q	q
(S)-proacaciberin	p	q	q
(R)-3-hydroxyheterodendrin	p	p	q
linamarin	n	q	q
(R)-lotaustralin	n	q	q
gynocardin	n	q	q

[a] Duplicate calibration curves for each glycoside, estimated against amygdalin standards by flying spot densitometry. 24 h of incubation at room temperature. I = 0.1% w/v β-glucosidase; II = 6.0% v/v β-glucuronidase; III = 0.3% w/v β-glucosidase plus 6% v/v β-glucuronidase. q = quantitative cleavage; p = partial cleavage; n = no cleavage.

heterodendrin [from *Acacia sieberana* D.C. var. *woodii* (Burtt Davy) Keay and Brenan] was hydrolyzed too slowly to give quantitative release of HCN within the time of incubation. The problem could be solved, however, by use of a mixture containing 0.3% w/v β-glucosidase and 6000 F units/mL β-glucuronidase as the spray reagent. The above mentioned results are summerized in Table I. Enzyme preparations from *H. pomatia* have previously been shown to catalyze the hydrolysis of sterically hindered glycosides (Brimer et al., 1982) and amygdalin (Giaja, 1919).

In order to support these results, the four crystalline glycosides amygdalin, linamarin, lucumin, and gynocardin were hydrolyzed in solution by glucuronidase. The released hydrogen cyanide was determined (microdiffusion) according to the method of Epstein as modified by Jørgensen (1955). In all cases, quantitative liberation of hydrogen cyanide was observed [recovery (%): amygdalin 107.6, linamarin 105.8, lucomin 99.9, and gynocardin 102.3; 2.7 μmol of substrate in 2 mL of an aqueous solution of β-glucuronidase (G 0751), 6000 F units/ mL, 18 h at 23–25 °C]. Similar experiments with β-glucosidase demonstrated the inability of this enzyme to cleave quantitatively linamarin and gynocardin [recovery (%): linamarin 0.0 and gynocardin 0.0; 2.7 μmol of substrate in 2 mL of 0.1% w/v β-glucosidase (almond), 0.066 M phosphate buffer (pH 6.0)].

The picrate test is known not to be entirely specific for HCN; thus, aldehydes, degradation products from gluco-
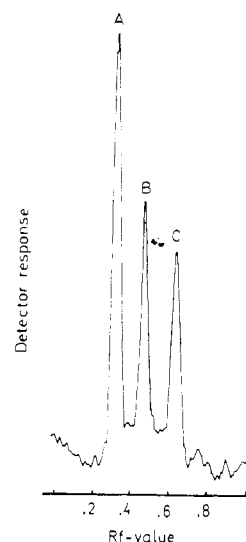

Figure 3. Densitometric recording of thin-layer chromatogram: (A) proacaciberin, 32.8 nmol; (B) 3-hydroxyheterodendrin, 15.4 nmol; (C) proacacipetalin, 8.6 nmol. Found: (A) 37.3 nmol; (B) 14.4 nmol; (C) 9.8 nmol. Si gel (precoated plates, Merck 5721); EtOAc–(CH$_3$)$_2$CO–CHCl$_3$–MeOH–H$_2$O (40:30:12:10:8); two ascents.

sinolates, and naphthoquinones have been reported to form orange-red products with picrate (Mitchell and Richards, 1978; Nahrstedt, 1980). Similar reactions were also observed in our system; thus, sinigrin was visualized as a diffuse orange-red spot after spray with myrosinase, whereas benzaldehyde yielded a red-violet spot. In both cases, however, greater amounts of compound was needed. Thus, the molar to molar ratios (giving the same color reaction) was found in the intervals sinigrin–amygdalin (12–20:1) and benzaldehyde–amygdalin (500–600:1). In the case of sinigrin, no reaction was observed after spray with glucuronidase.

In order to demonstrate the utility of the method, an artificial mixture of cyanogenic glycosides occurring in *A. sieberana* D.C. var. *woodii*, namely, proacacipetalin, 3-hydroxyheterodendrin, and proacaciberin, was separated by thin-layer chromatography, and the amount of cyanide liberated was determined against amygdalin standards (Figure 3). Thus, in contrast to the incubation method this procedure enables separate quantification of different cyanogenic constituents present in food or fodder, provided a TLC separation is obtained. As shown in Figure 4, the amounts of linamarin and lotaustralin in linseed meal could be estimated separately, whereas cochromatography of linustatin and neolinustatin only allowed the total amount of these two glycosides to be determined. The prepondenrance of linamarin in extracts from cassava meal (Dunstan et al., 1906) and lima beans (Butler, 1965) makes the signal from lotaustralin appear only as a shoulder on

Table II. HCN Released from Compounds in Extracts and from Crude Materials (μmol/g)[a]

source	TLC-densitometric analysis of extracts				hydrolysis in solution, total HCN	
	compound			total HCN (calcd)		
	linamarin/lotaustralin				extract	crude material
linseed meal[d]	4.6 ± 0.6 (n = 4)[b]	0.46 ± 0.05 (n = 2)	0.36 ± 0.09 (n = 2)	5.4 ± 0.7	4.7 ± 0.6 (n = 6)	5.1 ± 0.5 (n = 4)
"white" lima beans[d]	0.45 ± 0.1 (n = 3)[c]	1.35 ± 0.07 (n = 3)		1.8 ± 0.1	1.8 ± 0.2 (n = 4)	1.8 ± 0.2 (n = 4)
cassava tuber (root) meal[e]		0.95 ± 0.1 (n = 3)		0.95 ± 0.1	1.0 ± 0.15 (n = 4)	0.89 ± 0.09 (n = 4)

[a] Results given as mean ± SD; n equals the number of determinations; for extracts, on the same extract. [b] Linustatin plus neolinustatin. [c] Unknown compound(s). [d,e] Separated by the system Si gel (precoated plates, Merck 5721), EtOAc–(CH$_3$)$_2$CO–CHCl$_3$–MeOH–H$_2$O (40:30:12:10:8): (d) two ascents; (e) one ascent.

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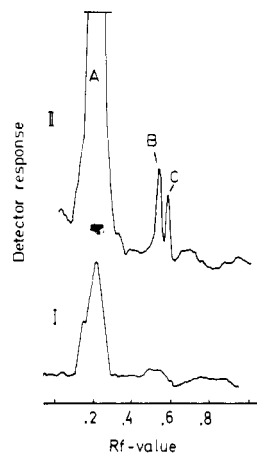
Figure 4. Densitometric recording of thin-layer chromatogram of an extract from linseed meal: 20.00 g extracted 3 times with 30 mL of boiling 80% EtOH. Pooled extracts were evaporated and the residue was dissolved in 30.00 mL of 80% ethanol. (I) 6.0 μL and (II) 20.0 μL. (A) Linustatin plus neolinustatin; (B) linamarin; (C) lotaustralin. Si gel (precoated plates, Merck 5721); EtOAc–(CH₃)₂CO–CHCl₃–MeOH–H₂O (40:30:12:10:8); two ascents.

the signal from linamarin. Consequently, a better TLC system is needed for separate quantitation of the two glucosides in these articles of food. As is evident from Table II, excellent agreements between the total cyanide contents as determined by the incubation or the TLC-densitometric methods are obtained.

The advantage of the TLC–densitometric method is illustrated by the observation that the extract of lima beans besides linamarin and lotoustralin contained a previously not observed cyanogenic principle cochromatographing with linustatin/neolinustatin. Further investigations as to the identity of this principle making up about 30% of the total cyanide content are in progress.

ACKNOWLEDGMENT

Registry No. β-Glucuronidase, 9001-45-0; (*R*)-prunasin, 99-18-3; (*S*)-sambunigrin, 99-19-4; (*R*)-amygdalin, 29883-15-6; (*R*)-holocalin, 41753-54-2; (*S*)-dhurrin, 499-20-7; triglochinin, 28876-11-1; (*S*)-proacacipetalin, 66871-89-4; (*S*)-heterodendrin, 66465-22-3; (*S*)-cardiospermin, 54525-10-9; (*S*)-cardiospermin sulfate, 85719-46-6; (*R*)-lucumin, 1392-28-5; (*S*)-proacaciberin, 79197-21-0; (*R*)-3-hydroxyheterodendrin, 80750-13-6; linamarin, 554-35-8; (*R*)-lotaustralin, 534-67-8; gynocardin, 14332-17-3.

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