ARTICLES

Anthocyanins from Bambara Groundnut (*Vigna subterranea*)

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Among five pigments detected in bambara groundnut (*Vigna subterranea*), three major anthocyanins were isolated by combined column chromatography on Amberlite XAD-7, Sephadex LH-20, and RP-18 silica gel and by preparative thin-layer chromatography on silica gel. They were identified as delphinidin 3-*O*-*β*-glucoside, petunidin 3-*O*-*β*-glucoside, and malvidin 3-*O*-*β*-glucoside on the basis of chromatographic data and UV−vis, ^{1}H-NMR, and mass spectrometry.

Keywords: *Vigna subterranea; Fabaceae; delphinidin; petunidin; malvidin; glucoside; ^{1}H NMR*

INTRODUCTION

The grains of *Vigna subterranea* [L.] Verdc. (Fabaceae) (Mabberley, 1987) are widely used as food in West Africa. Previous works on other *Vigna* species have led to the isolation of cyanidin, malvidin, and delphinidin glycosides (Mazza and Miniati, 1996). Delphinidin 3-glucoside was found in *V. mungo*, *V. radiata*, and *V. angulari*; cyanidin 3-glucoside was identified in *V. mungo* and *V. radiata*; delphinidin 3-glucoside was isolated from *V. angularis* and *V. radiata*; delphinidin 3-(*p*-coumaryl)glucoside was detected in *V. mungo* and *V. radiata*, while delphinidin 3,5-diglucoside was found only in *V. radiata*.

To complete the chemical investigation of the genus *Vigna* and to pursue our studies on the pigment content of several plants growing in Burkina Faso, we report here the identification of 3-*β*-*O*-monoglucosides of cyanidin, petunidin, and malvidin in *V. subterranea* grains.

MATERIALS AND METHODS

Reagents and Solvents. All were of analytical grade and obtained from Merck-Clevenot Corp., Darmstadt, Germany.

Sample Preparation. Extracts were obtained from grains of *V. subterranea* collected in 1995 in Burkina Faso, West Africa. The pigments were extracted twice by maceration of 600 g of the grains with 1% trifluoroacetic acid (TFA) in CH_3OH at 5 °C for 48 h. The crude extract was concentrated to dryness under vacuum and dissolved in 10 mL of 0.5% TFA in H_2O. This solution was filtered and fixed on a nonionic polymeric adsorbent (Amberlite XAD-7, Aldrich Chemical Co., Milwaukee, WI) column (length 300 mm, i.d. 20 mm) which was prewashed with 0.5% TFA/H_2O; the pigments were then eluted with MeOH/H_2O/TFA, 70:30:0.3). The eluate was concentrated, filtered through a Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) column (length 300 mm, i.d. 20 mm), and further purified on a Lobar glass prepacked column (Licroprep RP-8 column, 310 mm length, 25 mm i.d., Merck-Clevenot Corp.) with MeOH/H_2O/TFA, 40:60:0.3. The final purification was achieved by preparative thin-layer chromatography (TLC) on silica gel 60 F_{254} (Merck-Clevenot Corp.) using EtOAc/HCO_2H/HOAC/H_2O, 100:11:101:26 (EFAW), as solvent system. The isolated bands of adsorbent were eluted with 0.5% TFA/MeOH, and the solutions were concentrated and filtered through a RP-18 column using (0.5% TFA/H_2O)/MeOH (6:4). The eluates were finally concentrated and freeze-dried to give **1** (5 mg), **3** (10 mg), and **5** (4 mg) as TFA salts.

HPLC and GLC Conditions. High-performance liquid chromatography (HPLC) was carried out following the method described by Gao and Mazza (1994); the elution conditions were, however, modified according to the polarity of the pigments under investigation. The chromatographic system was equipped with two pumps (Model 6000 A), a solvent programmer (Model 660) from Waters Associates (Milford, MA), a diode array detector operating at 520 nm (Model 1040A from Hewlett-Packard, Germany), and a Nova Pak C_{18} column (150 × 3.9 mm; mean particle size = 4 μm); the elution was performed at 25 °C at a flow rate of 0.8 mL min^{-1}. A linear gradient solvent system was applied for 20 min from 20 to 60% of solvent B (MeOH/H_2O/HCO_2H, 75:24.5:0.5) in solvent A (H_2O/HCO_2H, 60:1); the final isocratic conditions for elution were maintained for 10 min. A photodiode array detector was used for the detection (520 nm) and the UV−vis spectra recording (from 250 to 600 nm).

Gas−liquid chromatography (GLC) identification of the sugar moities was carried out by dissolving 2 mg of each anthocyanin in 4 mL of 2 N TFA/H_2O and heating in a sealed vial at 110 °C for 45 min. The anthocyanidins were extracted twice by 0.5 mL of 3-methyl-2-butanol (Ribéreau-Gayon, 1968). The resulting aqueous fractions were evaporated to dryness. The residue was taken for silylation in pyridine/*N*-(trimethylsilyl)imidazole (2:1) and heated in a sealed vial at 60 °C for 15 min. The silylated derivatives of sugar residues were identified by GLC on a fused silica capillary column coated with CP-Sil-8-CB (length 25 m, i.d. 0.32 mm, film thickness 0.25 μm, Chrompack, Belgium) using cochromatography with sugar standards.

Spectroscopic Analysis. ^{1}H NMR spectral data were recorded on an instrument operating at 250 MHz (Model WM 250, Bruker, Karlsruhe, Germany) in CD_3OD/TFA-d_1 (0.5:0.1 mL) with TMS as internal standard. When the signals of the anomeric protons of the investigated anthocyanins were partially overlapped by the signal of the dissolved H_2O, an additional shift to lower magnetic field of this signal was produced by adding 30 μL of TFA-d_1.

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S0021-8561(96)00897-7 CCC: $14.00

Table 1. Chromatographic and UV−Vis Absorption Data of Anthocyanins 1, 3, and 5[a]

	TLC (R_f × 100)					HPLC	UV−vis data (in 0.01 N HCl−MeOH)			
anthocyanin	BAW[a]	BuHCl[a]	1% HCl[a]	AHW[a]	EAFW[b]	(R_t in min)	UV λ, nm (log ε)	vis λ, nm (log ε)	+$AlCl_3$[d] (Δ in nm)	$E_{440}/E_{viz,max}$
1	19	06	05	10	34	14.40	279 (4.14)	541 (4.47)	+14	16
3	25	07	05	14	39	17.10	278 (4.22)	540 (4.55)	+9	16
5	32	11	08	23	44	19.10	278 (4.14)	539 (4.43)		18

[a] Solvent systems: BAW, 1-butanol/acetic acid/water (4:1:5 upper phase); BuHCl, 1-butanol/2 N HCl (1:1 upper phase); 1% HCl, water/concentrated HCl (99:1); AHW, acetic acid/concentrated HCl/water (15:3:82); EAFW, ethyl acetate/acetic acid/formic acid/water (100:11:11:26). [b] Adsorbent: cellulose microcrystalline F. [c] Adsorbent: silica gel 60. [d] 2−3 drops of 5% $AlCl_3$ in MeOH were added.

Table 2. ^{1}H-NMR Spectral Data of Anthocyanins 1, 3, and 5 [δ in CD_3OD/TFA-d_1 (5:1)]

anthocyanin	**1** δ (*J* in Hz)	**3** δ (*J* in Hz)	**5** δ (*J* in Hz)
aglycon	delphinidin	petunidin	malvidin
H-4	8.98 s	8.89 s	8.94 s
H-6	6.68 d (1.9)	6.57 d (1.9)	6.60 d (1.9)
H-8	6.88 d (1.3)	6.78 d (1.3)	6.83 d (1.3)
H-2′	7.78 s	7.82 d (2.1)	7.87 s
H-6′	7.78 s	7.69 d (2.1)	7.87 s
O-CH_3		3.89 s	3.91 s
3-*O*-β-D-glucopyranose			
H-1″	5.32 d (7.6)	5.23 d (7.6)	5.25 d (7.6)
H-2″, H-3″, H-4″, and H-5″	3.51−3.81	3.26−3.68	3.30−3.71
H-6a″	3.96 dd (1.3, 10.4)	3.84 dd (1.2, 12.0)	3.84 dd (2.0, 12.0)
H-6b″	3.78 d (10.4)	3.66 d (12.0)	3.65 d (12.0)

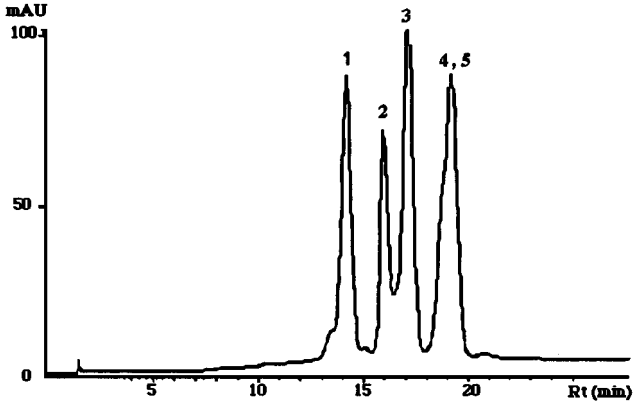
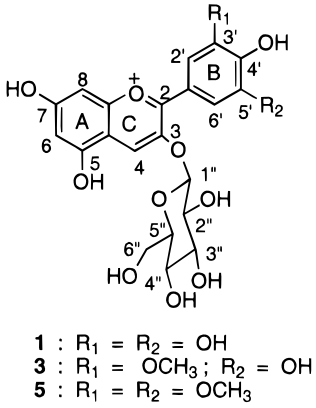


Figure 1. HPLC chromatogram of a crude methanol extract of grains of *V. subterranea* (conditions are given under Materials and Methods).



1 : R_1 = R_2 = OH
3 : R_1 = OCH_3 ; R_2 = OH
5 : R_1 = R_2 = OCH_3

Liquid secondary ion mass spectrometry (SIMS) was carried out using a mass spectrometer (Model Autospec, Micromass, Manchester, England). The matrix was glycerol/HCl (1:1).

RESULTS AND DISCUSSION

V. subterranea grains were extracted by methanol with added TFA. A preliminary HPLC of the crude extract allowed the detection of five pigments (Figure 1) having UV−vis spectra typical of the anthocyanins. The crude methanol extract was chromatographed successively on an ion-exchange resin, on a Sephadex column, and on a reversed phase silica gel column to give three major pigments: **1** (relative proportion = 26%), **3** (relative proportion = 23%), and **5** (relative proportion = 36%). They were finally purified by preparative TLC on silica gel. R_f data observed in standard TLC conditions used for this group of pigments, retention times in HPLC, and absorption maxima in UV−vis spectra of **1**, **3**, and **5** are presented in Table 1.

Our attemps to isolate the minor pigments **2** (relative proportion = 8%) and **4** (relative proportion = 4%) were unsuccessful, and their identifications were therefore dropped from of the study. The complete separation of **4** from **5** was only possible by preparative TLC on silica gel. All isolated pigments were present in the crude methanol extract as confirmed by comparison of the HPLC profiles of a crude methanol extract prepared without TFA; a possible hydrolysis during the extraction steps in the presence of TFA was thus discarded.

The hydrolysis of **1**, **3**, and **5** in TFA solution afforded glucose as confirmed by GLC analysis after silylation. The SIMS of anthocyanins **1**, **3**, and **5** showed strong $[M]^{+\bullet}$ ions at, respectively, *m*/*z* 465, 479, and 493, consistent with $C_{21}H_{21}O_{12}$, $C_{22}H_{23}O_{12}$, and $C_{23}H_{25}O_{12}$ molecular formulas. In addition, ions observed at *m*/*z* 303 for **1**, at *m*/*z* 317 for **3**, and at *m*/*z* 331 for **5** were assigned to a loss of $[C_6H_{10}O_5]^+$, the glucosyl moiety, in accordance with the GLC results. The linkage of the glucosyl moiety at C-3 was deduced from the chemical shift of the anomeric protons (Table 2) and by the $E_{440}/E_{vis,max}$ values (Table 1) (Ribéreau-Gayon, 1968). The β configuration of the glucose moiety of all three anthocyanins was confirmed from the magnitude (J = 7.6 Hz) of the $J_{1'',2''}$ coupling constant in the ^{1}H NMR spectra (Table 2). The ^{1}H NMR data related to the aglycon protons were in close agreement with those reported for other delphinidin, petunidin, and malvidin 3-*O*-β-glycosides (Kim et al., 1989; Terahara et al., 1990; Tsuda et al., 1994). The complete data, which were partially reported until now, are listed in Table 2.

Compounds **1**, **3**, and **5** were, therefore, respectively, delphinidin 3-*O*-β-glucoside, petunidin 3-*O*-β-glucoside,

and malvidin 3-*O*-β-glucoside. The coloration of different grain samples showed marked variations; therefore, the pigment concentration was assumed to be dependent on the cultivation region and on the harvest time. The usual consumption of the *V. subterranea* grains in Burkina Faso provided a mean daily intake of 5−10 mg of the pigments. Because of the known biological activities of anthocyanins (Morazzoni and Bombardelli, 1996), health benefits due to significant consumption of this food have to be considered.

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CAS Registry No. (Supplied by the Author): delphinidin 3-*O*-β-glucoside, 6906-38-03; malvidin 3-*O*-β-glucoside, 7228-78-6; petunidin 3-*O*-β-glucoside, 6988-81-4.

Received for review November 25, 1996. Revised manuscript received June 20, 1997. Accepted June 26, 1997.[⊗] This work was supported by a grant from the Fond Léopold Molle, Université Libre de Bruxelles, Belgium.

JF960897C

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.