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Simultaneous Determination of Furostanol and Spirostanol Glycosides in Taiwanese Yam (*Dioscorea* spp.) Cultivars by High Performance Liquid Chromatography

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ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of the steroid saponins, furostanol and spirostanol glycosides in Taiwanese yam cultivars. C18 column kept at 45°C was used as the stationary phase. The mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. Both evaporative light scattering detector (ELSD) and UV (203 to 215 nm) were employed and compared for the detection of saponins. The detection limits for ELSD and 203 nm UV were 2~3.5 mg and 0.1~0.2 μg, respectively at signal-to-noise ratio of 10. The recoveries of furostanol and spirostanol glycosides in the range of 0.25~3 μg were all above 92%. The presence of steroid saponins in Taiwanese yams was also determined.

Key words: diosgenin, evaporative light scattering detector, furostanol glycoside, high performance liquid chromatography, saponins, spirostanol glycoside, yam (*Dioscorea* spp.)

INTRODUCTION

Yam (*Dioscorea* spp.) is an important tuber plant for edible and medicinal use to promote health and longevity in Chinese tradition[1]. Saponin was reported to be the major physiologically active compound in yam[1-3]. It usually exists as glycoside in nature and many biological activities, such as hemolytic[4,5], hypocholesterolemic[6,7], hypoglycemic[8], anti-thrombotic[5,9], anti-neoplastic[2,3], anti-viral[10] and anti-cancer[11, 12] activities have been observed. Diosgenin, obtained from yam saponins after hydrolysis, is a principal starting material for industrial production of steroidal drugs[13,14].

Seven steroid saponins have been isolated from *Dioscorea colletti* var. hypoglauca[2,3]. They can be divided into two groups: (1) furostanol glycosides including protoneodioscin, protodioscin, protoneogracillin and protogracillin, (2) spirostanol glycosides including prosapogenin A of dioscin, dioscin and gracillin. There is, however, no report on the qualitative and quantitative determination of saponins in yam cultivars for routine work.

In the present investigation, we developed a high performance liquid chromatographic (HPLC) method for the simultaneous determination of the steroid saponins, including furostanol and spirostanol glycosides. Both UV and evaporative light scattering detector (ELSD) were employed and compared for the detection of saponins. Taiwanese yam cultivars were used as reference samples for testing method evaluation and modification.

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MATERIALS AND METHODS

I. *Material*

Tubers of three yam cultivars were collected in 2001 and 2002 from different areas of Taiwan. Two tubers belonged to *Dioscorea alata* L. The common names and the characteristics (shape of tuber/ color of cortex/ flesh) of these tubers were Ja-I yam (cylindrical/ white/ white) and Yang-Ming-Shan yam (cylindrical/ white/ white). One of the tubers belonged to *D. pseudojaponica* Yamamoto, which was commonly called Kee-Lung yam and was thin cylindrical in shape with white cortex and flesh. Ja-I yam was purchased from a farm in Hualien County. Yang-Ming-Shan yam was purchased from a farm near Yang-Ming-Shan National Park (Taipei City) and Kee-Lung yam was purchased from Chidu in Keelung City. Yam tubers were cleaned, peeled and cut into 4 mm slices using Salad Shooter (National Presto Industries, Eau Claire, WI, USA). The slices were freeze-dried by the FreeZone 18L Freeze Dry System (Labconco Co., Kansas City, MO, USA). All of the freezedried yam slices were ground with a grinder (model RT08, Rong-Tsong Co., Taipei, Taiwan). Steroid saponin standards, including three furostanol glycosides and three spirostanol glycosides were prepared in our laboratory from Kee-Lung cultivar of Taiwanese yam. The method was based on that reported by Chludil et al.[15] and DeMarino et al.[16] The furostanol glycosides were 26-*O*-β-D-glucopyranosyl-22α-methoxyl-25-(*R*)-furost-5-en-3β, 26-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[β-L-rhamnopyranosyl-

(1→4)]}-β-D-glucopyranoside (fu1), methyl protodioscin (fu2) and methyl protogracillin (fu3). The spirostanol glycosides were 25(*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside (sp1), dioscin (sp2) and gracillin (sp3). Structures of the saponins are shown in Figure 1. The purities of these standards were above 95% as determined by % total peak area on HPLC chromatogram using ELSD detector. Solvents used for the extraction and analysis of steroid saponins, including methanol, n-butanol, chloroform and sulfuric acid, were purchased from Tedia Co. (Fairfield, OH, USA). Deionized water was obtained from Milli-Q water purification system (Millipore Co., Bedford, MA, USA). It was degassed under vacuum and filtered through a 0.2 μm membrane filter prior to use in the HPLC analysis. *p*-Dimethylaminobenzaldehyde (for Ehrlich reagent) was purchased from Sigma Co. (St. Louis, MO, USA).

II. *HPLC Analysis of Six Steroid Saponins Standards*

Analyses were performed with a Hitachi L-7100 HPLC pump (Hitachi Instruments Inc., Tokyo, Japan) using Luna C18 column (4.6 mm i.d. × 250 mm, 5 μm) (Phenomenex, Torrance, CA, USA) as the stationary phase, which was kept at 45°C in a Colbox column oven (Hipoint Scientific Co., Kaohsiung, Taiwan). The mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. Either Alltech ELSD 2000 evaporative light scattering detector (ELSD) (tube temperature, 95°C; air flow rate, 2.8 L/min) (Alltech Associates Inc., Deerfield, Ireland) or Hitachi L-7420 UV/VIS detector (Hitachi Instruments Inc., Tokyo, Japan) was used to detect these steroidal saponins. The HPLC separation efficiency was evaluated by the separation factor (α) and resolution (Rs). The maximum wavelength

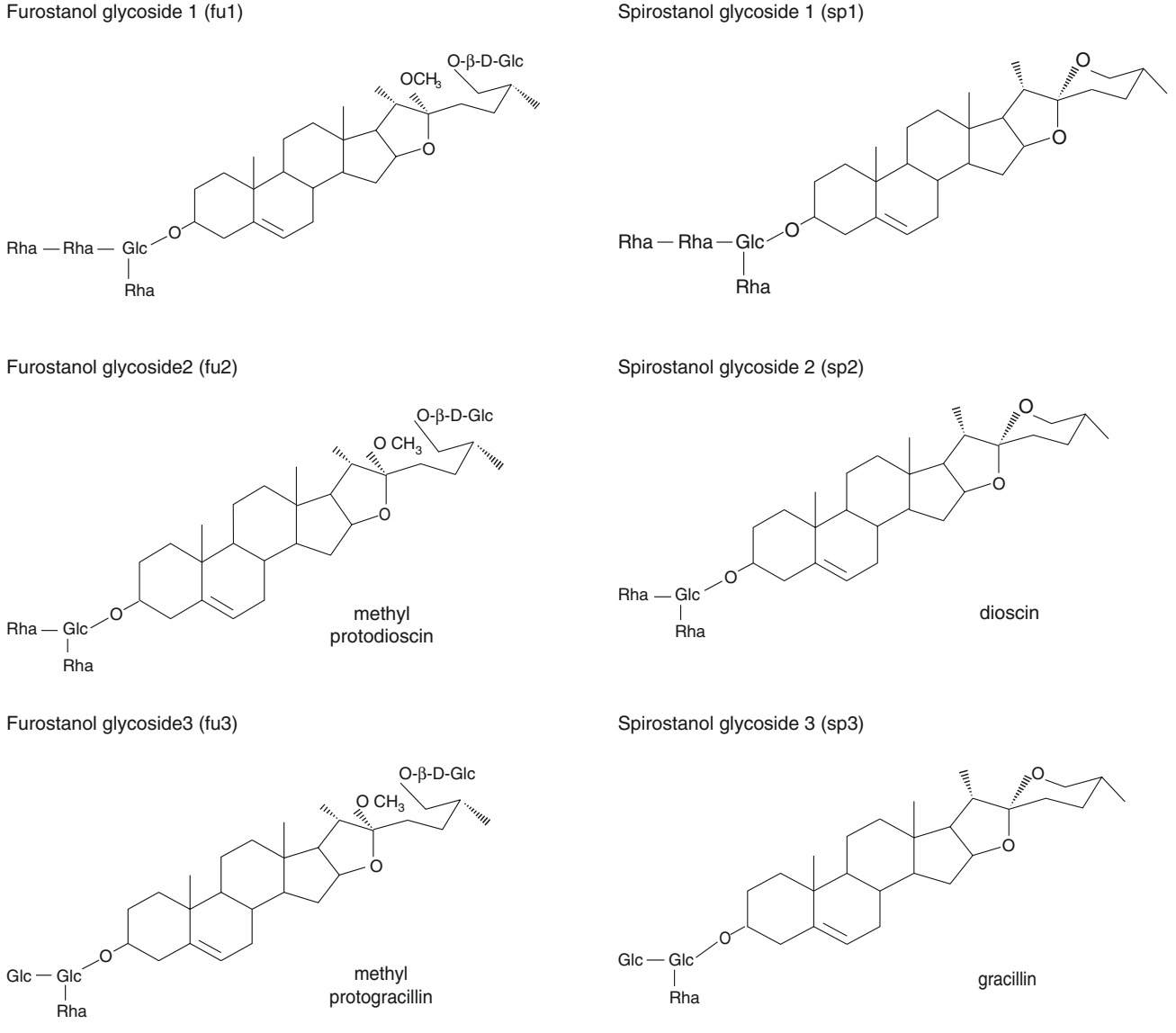

Figure 1. Structures of furostanol glycosides and spirostanol glycosides prepared from *D. pseudojaponica* Yamamoto.

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for the UV/VIS absorption of the six steroid saponins was determined by a Hitachi-U3210 spectrophotometer (Hitachi Instruments Inc., Tokyo, Japan). The detection limit of each steroid saponin was determined as the minimum sample concentration which gave the signal-to-noise ratio (S/N) of 10.

Six concentrations of each steroid saponin standards were injected at 20 μL into HPLC (the ranges for UV 203 nm and ELSD detection are described in Table 3), and the linear regression equation of the standard curve for each steroid saponin was obtained by plotting the amount of saponin injected against the peak area. The regression equation and the correlation coefficient (r^2) were calculated using CHEM-WIN computer software system (Shuen-Hua Co., Taipei, Taiwan). The standard curves of all steroid saponins were determined in triplicate and the mean values were calculated.

III. *HPLC Analysis of Saponins in Yam*

Steroid saponins were extracted from the three cultivars of yams with method similar to that reported in the literatures[2,3,17]. Freeze-dried yam powder (100 g) was extracted with 1 L of methanol for 24 hr at 25°C followed by filtration and concentration in the Rotavapor RE111 rotary evaporator (Büchi Co., Flawil, Switzerland). The residue was suspended in 25 mL of distilled water and partitioned against 25 mL of n-butanol for three times to yield saponin extract. The extract was washed with 50 mL of distilled water for three times and then concentrated to dryness by evaporating the solvent in the rotary evaporator. The dried crude extract was dissolved in 1 mL of methanol prior to the quantitative determination of steroid saponins by HPLC.

Yam extracts (20 μL) were injected into HPLC and analyzed under the same condition as the saponin standards. The steroid saponins in yam extracts were identified by: (1) comparison of retention time and mass spectrum (fu 1, M.W. = 1208; fu 2, M.W. = 1062; fu 3, M.W. = 1078; sp1, M.W. = 1014; sp 2, M.W. = 868; sp 3, M.W. = 884) obtained on a VG platform II LC-MS (Micromass Co., Cheshire, UK). Conditions of LC-MS: ESI^+ mode, cone voltage = 40 eV, source temperature = 200°C; (2) co-chromatography of furostanol and spirostanol glycoside standards with sample; (3) confirmation by thin layer chromatography (TLC). A TLC method similar to that developed by Konishi et al.[18] and Pierre et al.[19] was used. The yams extracts were spotted on silica gel 60 F_{254} TLC plate (No.1.05715, Merck Co., Darmstadt, Germany) and developed with $CHCl_3/CH_3OH/H_2O$: (8/4/1, v/v/v). Visualization was carried out with two spraying agents: (1) Ehrlich reagent (3.2 g *p*-dimethylaminobenzaldehyde in 60 mL 95% ethanol and 60 mL 12N HCl) and heated at 110°C for 5 min after spraying. Furostanol glycosides would show bright red color, while spirostanol glycosides did not show any color change; (2) 10% sulfuric acid (in methanol) and heated at 110°C for 5 min after spraying. In this case, furostanol and spirostanol glycosides were all yellow-green colored.

The recoveries were determined by adding mixture of these six steroid saponin standards (each weighing 0.25, 0.5, 1 and 3 mg) to 100 g of freeze-dried Kee-Lung yam (*D. pseudojaponica* Yamamoto) powder and extracted as

Table 1. Separation factor (α) and resolution (Rs) of the six steroid saponins standards

Compound	Peak number		α[a]	Rs[b]
Furostanol glycoside	1	fu1	–	–
	2	fu2	1.085 (fu1/fu2)[c]	1.247 (fu1/fu2)
	3	fu3	1.141 (fu2/fu3)	1.851 (fu2/fu3)
Spirostanol glycoside	1	sp1	–	–
	2	sp2	1.046 (sp1/sp2)	1.613 (sp1/sp2)
	3	sp3	1.042 (sp2/sp3)	1.395 (sp2/sp3)

[a]$\alpha = t_{R2}\text{-}t_0 / t_{R1}\text{-}t_0$, where t_{Rn} = retention time of an analyte, t_0 = retention time of an unretained peak.

[b]$Rs = 2\ (t_{R2}\text{-}t_{R1}) / (w_1+w_2)$, where w_n = band width of an analyte at the baseline.

[c]Values in parentheses represent two neighboring peaks.

Table 2. UV λ_{max} and the detection limits of the six steroid saponins standards using UV and ELSD detections

Steroidal saponins		UV λ_{max} (nm)	Detection limit (μg)[a]				
			UV				ELSD
			203 nm	208 nm	214 nm	215 nm	
Furostanol glycoside	1	202.0	0.1	0.5	1	1	2
	2	202.4	0.1	0.5	1	1	2
	3	202.4	0.2	0.75	1.5	1.5	2.5
Spirostanol glycoside	1	201.6	0.2	0.75	1.5	1.5	3.5
	2	201.2	0.1	0.5	1	1	2
	3	202.0	0.2	0.75	1.5	1.5	3

[a]Detection limit determined at S/N = 10.

Table 3. The linear regression equations of the six steroid saponins standards using UV 203 nm and ELSD detections

Compound		UV 203 nm			ELSD		
		Linear range (μg)	Linear regression equations[a]	Correlation coefficient (r^2)[a]	Linear range (μg)	Linear regression equations[a]	Correlation coefficient (r^2)[a]
Furostanol glycoside	1	0.1~60	Y = (8.35 X- 3.84) E4[b]	0.9994	2~60	Y = (1.76 X - 5.33) E4	0.9981
	2	0.1~60	Y = (10.91 X- 6.49) E4	0.9993	2~60	Y = (2.03 X - 5.29) E4	0.9984
	3	0.2~60	Y = (7.14 X- 2.71) E4	0.9996	2.5~60	Y = (1.13 X - 2.59) E4	0.9982
Spirostanol glycoside	1	0.2~60	Y = (6.07 X- 3.02) E4	0.9997	3.5~60	Y = (0.53 X - 1.83) E4	0.9988
	2	0.1~60	Y = (18.42 X- 4.65) E4	0.9996	2~60	Y = (2.44 X - 7.89) E4	0.9984
	3	0.2~60	Y = (10.54 X- 4.67) E4	0.9997	3~60	Y = (0.76 X - 2.41) E4	0.9984

[a]All data are the means of triplicate analyses.

[b]Y is the value of the peak area, X is the value of sample quantity (μg).

described above. The extracts were then subjected to HPLC analysis for steroid saponins contents, and then recoveries were calculated. The quantitative analyses and determination of recoveries of all steroid saponins were conducted in triplicate and the mean values were determined.

RESULTS AND DISCUSSION

I. *HPLC Separation of Six Steroid Saponins Standards*

Due to the polar nature of glycosidated saponins, high performance liquid chromatography (HPLC) is usually chosen for quantitative analysis. Steroid saponins lack remarkable chromophore for UV and visible detection, so the refractive index (RI) detection and the low-wavelength ultraviolet detection were used in HPLC analysis. Chen et al.[20] has employed a C18 column, a binary solvent system consisting of methanol and water, and RI detection to separate five spirostanol glycosides (the aglycone part was diosgenin) from *Paris* plants simultaneously. The nine spirostanol glycosides present in *Rhizoma paridis* were separated with a C8 column, which was a binary solvent system consisting of acetonitrile and water, and UV 203 nm detection. Five of them contained diosgenin in their aglycone parts[21]. In order to overcome the limit of the gradient elution using RI detection or low-wavelength ultraviolet detection, evaporative light scattering detector (ELSD) has been used to determine the triterpenoid saponins, ginsenosides[22].

We refered to these methods first. After repeated experiments and modification, we employed a C18 column and kept it at 45°C as the stationary phase. The mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. The three furostanol and three spirostanol glycosides could be separated simultaneously (Figure 2). The furostanol glycosides were eluted at about 20 min, while spirostanol glycosides were eluted at about 60 min. Table 1 shows the separation factors (α) and resolutions (Rs) of the saponins. The α values between fu1 and fu2, fu2 and fu3 were 1.085, 1.141 and the Rs values between fu1 and fu2, fu2 and fu3 were 1.247, 1.851, respectively. Those for the three spirostanol glycosides were 1.046, 1.042 and 1.613, 1.395, respectively. Therefore, the HPLC condition developed in this study showed good separation efficiency of the three furostanol glycosides and the three spirostanol glycosides on one chromatogram. This has not been reported previously. It can be used to determine the steroid saponins in yams for routine work.

UV spectra of the six yam steroid saponins were obtained by dissolving the standard compounds in methanol. The maximum absorption wavelengths of furostanol glycoside 1, 2 and 3 were 202.0, 202.4 and 202.4 nm, respectively, while those of spirostanol glycoside 1, 2 and 3 were 201.6, 201.2 and 202.0 nm, respectively (Table 2). We employed both UV and ELSD detectors during the HPLC analyses of yam saponins and compared their detection ability. The detection limits of the six steroid saponins were determined by ELSD and UV at 203, 208, 214 and 215 nm at signal-to-noise ratio (S/N) of 10 under the developed elution condition. Table 2 shows that the detection limits for 203 nm were 0.1~0.2 mg, which had the highest sensitivity among all UV detections. On the other hand, the detection limits for ELSD were 2~3.5 μg, which had a lower sensitivity compared to UV detection. However, there were more interferences and apparent baseline shift following solvent gradient elution during the determination of the steroid saponins with low-wavelength ultraviolet detection. ELSD could tolerate the interferences of impurities and the gradient elution of mobile phase, and therefore it would improve the accuracy for saponins determination (Figure 2 and 3).

Solutions containing 0.1~60 μg and 2~60 μg of the six saponin standards for UV 203 nm and ELSD detection respectively, were used to obtain standard calibration curves. They were linear and reproducible. The linear regression equations are shown in Table 3. The correlation coefficient (r^2) for UV 203 nm and ELSD detection were above 0.999 and 0.998.

II. *Determination of Steroid Saponins in Yam*

Extraction of steroid saponins in yams usually used methanol or ethanol. After evaporating alcohol, the residue was suspended in water and partitioned against n-butanol to extract saponins for analysis[2, 3, 17]. This method was adopted in our experiment. The freeze-dried yam powder was extracted with methanol followed by liquid-liquid extraction with water and n-butanol. The abovementioned HPLC method developed for the simultaneous analysis of six steroid saponins was employed. Table 4 shows that all

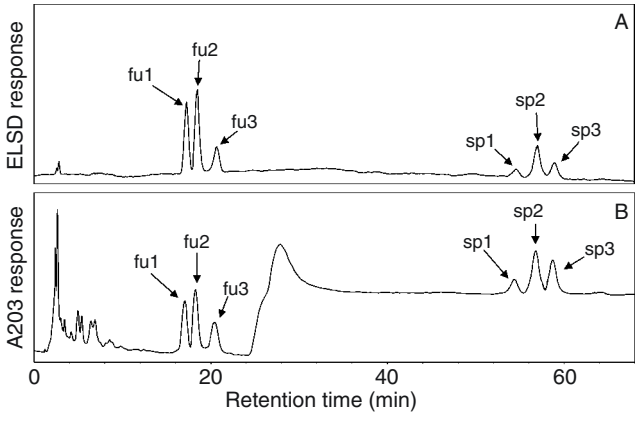

Figure 2. HPLC chromatograms of six steroid saponins. HPLC conditions: column, Luna C18 (4.6 mm i.d. × 250 mm, 5 μm); column temperature, 45°C; mobile phase, CH_3OH/H_2O = 62/38 from 1 to 20 min; 71/29 from 21 to 65 min; flow rate = 1 mL/min; detection, ELSD (tube temperature, 95°C; air flow rate, 2.8 L/min) (A) and 203 nm (B).

recoveries of the furostanol and spirostanol glycosides (each weighing 0.25 to 3 mg) were above 92%, regardless of the type and the amount of saponin added.

Figure 3 shows the HPLC chromatograms of the methanol extract of *Dioscorea pseudojaponica* Yamamoto. It was clearly indicated that the extraction and analytical methods established in this study could be successfully applied to the analysis of steroid saponins (furostanol and spirostanol glycosides) in Taiwanese yam cultivars without interference peaks, especially using ELSD. Thin layer chromatography (TLC) was also employed to assist in confirming the presence of steroid saponins. The R_f values for furostanol and spirostanol glycosides were around 0.44 and 0.67, respectively (data not shown). Table 5 shows individual content of the six steroid saponins in the three Taiwanese yam cultivars. Furostanol glycosides were found to be the major saponins in Kee-Lung yam and Yang-Ming-Shan yam. The two cultivars of *D. alata* L., Ja-I yam and Yang-Ming-Shan yam collected from different areas of Taiwan, had quite different steroid saponin composition. Dinan et al.[17] indicated that saponins content depended on many factors, e.g., age, geographic location of the plant and the cultivation conditions. It is assumed that these factors caused the difference in the amounts of furostanol and spirostanol glycosides in Ja-I yam and Yang-Ming-Shan yam. Chen and Wu[13] reported that diosgenin contents of the major *Dioscorea* species (*D. colletti*, *D. althaeoides*, *D. colletti* var. hypoglauca, *D. gracillima*, *D. nipponica*, *D. panthaica*, *D. parviflora* and *D. zingiberensis*) in China were 1.5~3.6% of fresh weight. In Indian yams, the contents of diosgenin were 1.42, 3.48 and 0.62% of dry weight in *D. floribunda*, *D. deltoidea* and *D. prazeri*, respectively[23]. However, contents of saponins in the glycoside forms, e.g., furostanol, spirostanol glycosides in yams, have not been reported yet.

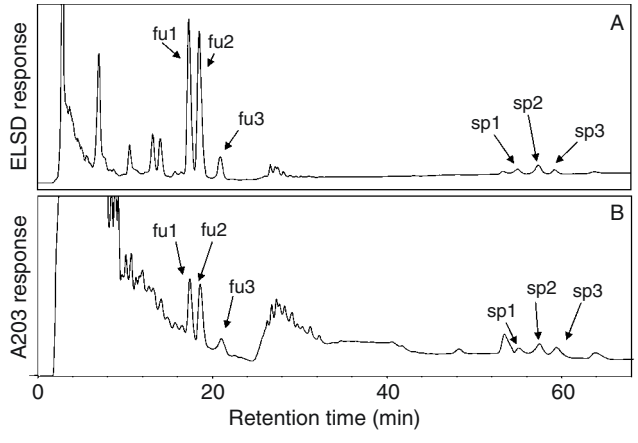

Figure 3. HPLC chromatograms of the methanol extract of *Dioscorea pseudojaponica* Yamamoto. HPLC conditions: same as in Figure 2.

In this study, the total saponin contents in the three Taiwanese yams were found to be in the range of 114.48 to 216.66 μg/g dw. Assuming saponins could be completely converted to diosgenin in acid hydrolysis, the diosgenin content in the three Taiwanese yams would be around 49.06, 57.55 and 84.41 μg/g dw. It is therefore evident that these Taiwanese yams had much lower diosgenin content than Chinese and Indian yams.

CONCLUSION

The three furostanol and three spirostanol glycosides

Table 4. The recovery (%) of added steroid saponins

Compound		Recovery (%)[a]			
		Amount of saponin added (mg)			
		0.25	0.5	1	3
Furostanol glycoside	1	92.57 (7.38)[b]	93.32 (3.74)	94.11 (3.24)	93.89 (1.96)
	2	93.62 (6.54)	94.06 (7.18)	94.52 (1.68)	94.58 (2.52)
	3	92.84 (4.12)	94.13 (8.06)	93.43 (3.06)	93.96 (1.73)
Spirostanol glycoside	1	93.25 (5.17)	93.16 (5.38)	94.16 (3.38)	94.35 (2.06)
	2	92.75 (2.48)	94.21 (3.66)	94.74 (2.66)	94.71 (3.17)
	3	93.28 (6.92)	93.66 (2.34)	93.66 (2.68)	94.25 (1.27)

[a]All data are the means of triplicate analyses.
[b]Values in parentheses are the coefficient of variation (%).

Table 5. Steroid saponins contents in yam fleshes (*Dioscorea* spp.)

Curtivars		Steroidal saponins contents (μg / g dw)[a]								
		Furostanol glycoside				Spirostanol glycoside				Total
		1	2	3	Total	1	2	3	Total	saponins
D. alata L.	Ja-I	10.44 ± 3.22	13.43 ± 2.58	10.94 ± 4.04	34.81	24.37 ± 3.12	34.23 ± 1.69	21.07 ± 3.53	79.67	114.48
	Yang-Ming-Shan	30.49 ± 3.57	36.57 ± 2.19	30.52 ± 1.85	97.58	8.03 ± 3.14	12.09 ± 4.03	25.88 ± 2.33	46.00	143.58
D. pseudojaponica Yamamoto.	Kee-Lung	49.57 ± 2.48	50.74 ± 2.78	47.42 ± 4.96	147.73	20.72 ± 2.87	27.37 ± 2.12	20.84 ± 2.95	68.93	216.66

[a]All values are mean ± S. D. obtained by triplicate analyses.

present in yam could be separated simultaneously by reversed-phase high performance liquid chromatography. C18 column kept at 45°C was used as the stationary phase, the mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. The recoveries of furostanol and spirostanol glycosides were above 92%. In the three Taiwanese yam cultivars, the contents of furostanol glycosides were found to range from 34.81 to 147.73 μg/g dw, while the contents of spirostanol glycosides ranged from 46 to 79.67 μg/g dw.

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