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Analytical Methods

Determination of steroidal saponins in different organs of yam (*Dioscorea pseudojaponica* Yamamoto)

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Abstract

Yams (*Dioscorea* spp.) are perennial trailing rhizome plants. Steroidal saponins, furostanol and spirostanol glycosides are the marked functional compounds in yams. In this investigation, a C18 solid phase extraction method was developed for yam saponins purification. The contents of saponins in various organs of yam (*Dioscorea pseudojaponica* Yamamoto) were also determined. Results showed that the recoveries of yam saponins extracted by the developed method were about 99.48–100.08% when the saponins (each saponin weighed 0.20, 0.50 and 1.00 mg) passing through the C18 cartridge. The extractive method could efficiently reduce the interferences from impurities in yam saponin extracts prior to HPLC analysis. The recoveries of added saponins in different yam organs were 98.34–99.92% for tuber flesh, 95.98–98.89% for tuber cortex, 97.89–99.44% for rhizophor, 93.82–98.01% for leaf and 93.87–97.65% for vine, respectively. The yam tuber cortex had the highest amount of saponins (582.53 μg/g dw), which was higher than that existed in the tuber flesh (227.86 μg/g dw) about 2.55 times. The contents of saponins in the rhizophor, leaf and vine of yam were 29.39, 24.41 and 23.96 μg/g dw, respectively.

Keywords: Furostanol glycoside; HPLC; Solid phase extraction; Spirostanol glycoside; Steroidal saponin; Yam (*Dioscorea* spp.)

1. Introduction

Yams (*Dioscorea* species) are perennial trailing rhizome plants, which belong to *Dioscoraceae* family. The crops serve as an important staple food in many parts of the world (Hariprakash & Nambisan, 1996). Wanasundara and Ravindran (1994) indicated that the yam tubers could be source of energy, primarily, as their dry material predominantly consists of carbohydrate (69.9–77.5%); moreover, the crude protein, crude fat, crude fiber and ash contents of yams are in the range of 6.7–7.9%, 1.0–1.2%, 1.2–1.8%, 2.8–3.8%, respectively. Furthermore, yam tubers contain vitamin C (13.0–24.7 mg/100 g dw) (Wanasundara & Ravindran, 1994), musin (glycoprotein) (2.11 g/100 g dw) (Wanasundara & Ravindran, 1992), minerals (K, P, Ca, Mg, Fe, Cu, Co) (Agbor-Egbe & Treche, 1995), phytosterols (sitosterol, stigmasterol and campesterol) (Šavikin-Fodulovic, Grubisic, Culafic, Menkovic, & Ristic, 1998) and steroidal saponins (furostanol and spirostanol glycosides) (Hu, Yao, Kobayashi, & Iwasaki, 1997; Yang, Lu, & Hwang, 2003a, 2003b), etc. They have been used widely in traditional Chinese medicine to promote human health and provided for functional foods producing in Taiwan (Liu, Wang, Shyu, & Song, 1995).

Steroidal saponins are the most noticeable bioactive compounds that show many biological functions, e.g., anti-carcinogenic (Ravikumar, Hammesfahr, & Sih, 1979; Sung, Kendall, & Rao, 1995), anti-thrombotic (Peng et al., 1996; Zhang et al., 1999), anti-viral (Aquino et al., 1991), hemolytic (Santos et al., 1997; Zhang et al., 1999), hypocholesterolemic (Malinow, 1985; Sauvaire, Ribes,

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Baccou, & Loubatieŕes-Mariani, 1991) and hypoglycemic (Kato, Miura, & Fukunaga, 1995). Diosgenin, the aglycone (sapogenin) of the yam steroidal saponins, is also used as the raw material for industrial production of steroidal drugs (Djerassi, 1992; Chen & Wu, 1994).

Dinan, Harmatha, and Lafont (2001) indicated that considerable difference of saponin contents (mainly quantitative) could be observed in plant organs. There were, however, no thorough reports on the variation of saponin contents in different organs of yam plants. For the tuber flesh of yam (*Dioscorea pseudojaponica* Yamamoto), we separated three furostanol glycosides and three spirostanol glycosides in the previous work (Yang et al., 2003a, 2003b). The tuber cortex, rhizophor, leaf and vine of yam may contain saponins as well; nevertheless, they are usually discarded after yam tuber was harvested and utilized. In the investigation, we developed a C18 solid phase extraction (SPE) method for yam saponin purification; moreover, the contents of saponins in various organs of yam were also determined.

2. Experimental

2.1. Materials

The Taiwanese native yam plants (*D. pseudojaponica* Yamamoto) including tubers with a white cortex and flesh (ca. 4 cm in diameter and ~120 cm long), leaves, vines and rhizophores (ca. 1 cm in diameter and ~4 cm long) were obtained from Keelung City, Taiwan on January 2006. The tubers were peeled and the cortices were collected. The flesh of yam tubers and rhizophores were cut into 4 mm thick slices. The leaves were separated from yam vines and then the vines were cut into 2 cm of length. All of the samples were lyophilized with the FreeZone 18L Freeze Dry System (Labconco Co., Kansas City, MO, USA) and ground to 40 mesh or below prior to use. Steroidal saponin standards (Fig. 1), furostanol glycosides including 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*-[α-L-rhamnopyranosyl-(1 → 4)]}-β-D-glucopyranoside (**1**), methyl protodioscin (**2**) and methyl protogracillin (**3**), and spirostanol glycosides including (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-{[α-L-rhamnopyranosy-(1 → 4)]-*O*-[α-L-rhamnopyranosyl-(1 → 4)]}-β-D-glucopyranoside (**4**), dioscin (**5**) and gracillin (**6**), were isolated as that reported in our previous study (Yang et al., 2003a). All of their purities were over 95% (Yang et al., 2003b). Solvents used for extraction and separation of steroidal saponins, *n*-butanol (*n*-BuOH) and methanol (MeOH), were purchased from Tedia Co. (Fairfield, OH, USA). Deionized water (H_2O) was prepared by Ultrapure™ water purification system (Lotun Co. Ltd., Taipei, Taiwan); it was degassed under vacuum and filtered through a 0.2 μm membrane filter (Millipore Co., Bedford, MA, USA) before use.

2.2. SPE of yam saponins

The SPE method was based on that reported by Yang et al. (2003b). Zorbax SPE C18 cartridges (200 mg) (Agilent Technologies Inc., Palo Alto, CA, USA) were conditioned by passing through 3 mL of MeOH followed by 3 mL of 25% MeOH (prepared with H_2O). Mixture of the six saponins standards (each weighed 1 mg) was dissolved in 1 mL of MeOH and then diluted with 3 mL of H_2O. The saponin solution was loaded onto a Zorbax SPE C18 cartridge followed by elution with 3 mL aliquots of 25–95% aqueous MeOH (in the increment of 5% each time) and 100% MeOH. Each eluate was collected and analyzed by HPLC in order to find the best eluting solvent for saponins.

2.3. Recoveries of yam saponin standards after SPE

Mixtures of the six saponin standards (each saponin weighed 0.20, 0.50 and 1.00 mg), dissolved in 1 mL of MeOH and then diluted with 1 mL of H_2O, were loaded onto Zorbax SPE C18 solid phase extraction cartridges. The cartridges were washed using 3 mL of H_2O followed by 3 mL of 50% MeOH (in H_2O) for the removal of possible interference matrix; the saponins were eluted with 3 mL of 80% MeOH (in H_2O) later. Each eluate was concentrated to dryness in a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) at 35 °C and re-dissolved in 1 mL of MeOH for HPLC analysis. The recovery of each saponin was calculated from the analytical result and the original quantity of saponin used.

2.4. Extraction efficiency of saponins from varied yam organs

The method used was modified from that reported by Yang et al. (2003b). Adding a mixture of the six saponin standards (each weighed 0.20, 0.50 or 1.00 mg) to 50 g of each yam sample (various organs), and subsequently extracted with 1 L of MeOH for 24 h at 25 °C. The MeOH extract was filtered and removed solvent in a rotary evaporator at 30 °C. The residue was suspended in 25 mL of distilled H_2O and partitioned against 25 mL of *n*-BuOH for three times to yield saponin extract. The extract was washed with 50 mL of distilled H_2O for three times and *n*-BuOH was taken away in a rotary evaporator at 45 °C afterward. The dried extract was dissolved in 1 mL of MeOH and added 1 mL of H_2O later. The follow-up treatment was done with Zorbax SPE C18 solid phase extraction as described above. The eluate was then subjected to HPLC analysis of saponins contents. The recoveries were calculated from the analytical result subtracting the original saponin amount in sample, and then divided by the amount of saponin added.

2.5. HPLC analysis of yam saponins

A *PrimeLine*™ Gradient Model 500G HPLC pump system (Analytical Scientific Instruments Inc., El Sobrante,

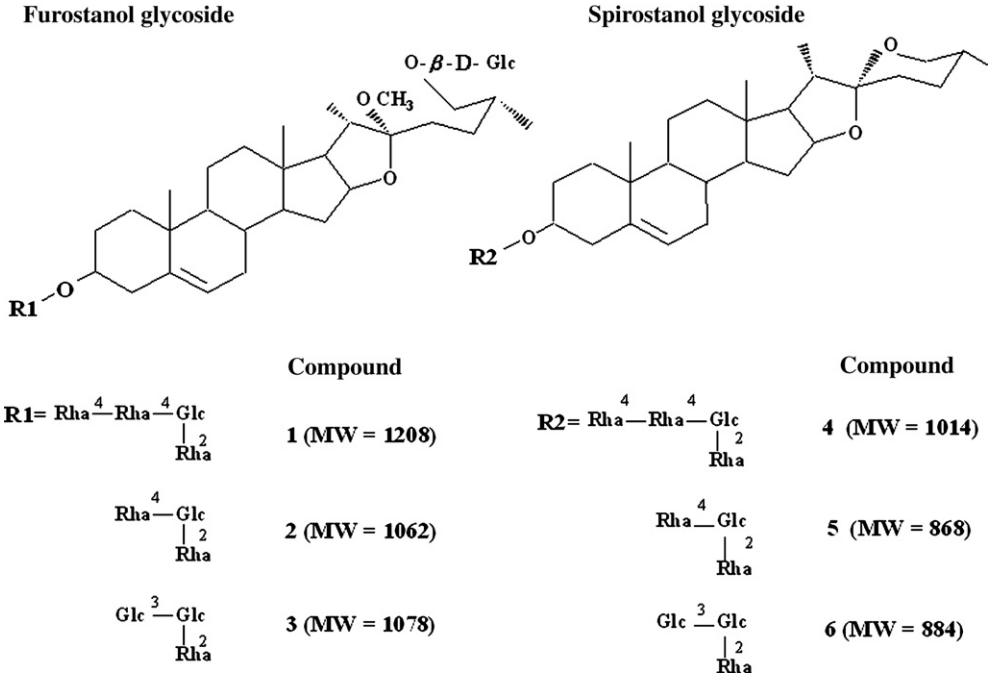

Fig. 1. Chemical structures of yam saponins.

CA, USA) with a 20 μL injection loop, equipped with an Alltech ELSD 3300 evaporative light scattering detector (ELSD) (tube temperature, 75 °C; air flow rate, 2.8 L/min) (Alltech Associates Inc., Deerfield, Ireland), was employed for yam saponins analysis. A revered-phase column (Luna C18 column; 4.6 mm i.d. × 250 mm, 5 μm particle size) (Phenomenex, Torrance, CA, USA) was used for separation. Elution was performed with a step gradient solvent system consisting of MeOH and deionized H_2O, 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 (Yang et al., 2003b). A ChemWin computer software system (Shuen-Hua Co., Taipei, Taiwan) was exploited for data processing.

2.6. Method validation

The intra- and inter-assay variabilities for all yam saponins were measured at concentrations of 0.20, 0.50 or 1.00 mg/mL from six measurements on the same day and six sequential days, respectively. The accuracy (% bias) was determined from the nominal concentration (C_n) and the mean value of observed concentration (C_o) as follows: % bias $= [(C_o - C_n)/C_n] \times 100$. The precision relative standard deviation (RSD) was calculated from the C_o as follows: % RSD = [standard deviation (SD)/C_o] × 100.

2.7. Statistical analysis

The recoveries and quantitative analyses of the yam saponins were conducted in triplicate and the mean values were determined. Statistical analyses of the data were executed by the analysis of variance and Duncan's test procedures were applied to estimate significance between means, at a level of $p < 0.05$.

3. Results and discussion

3.1. The method validation for yam saponin analysis

The HPLC method developed by Yang et al. (2003b) was adopted for the yam saponins separation. We estimated the method validation further while the study was carried out. The intra- and inter-assay variability on the same day and six sequential days for each yam saponin at 0.20–1.00 mg/mL were surveyed. Table 1 shows that RSDs of retention times of the six saponins were less than 1% for run-to-run and under 2% for day-to-day, respectively. Table 2 presents the precision (RSD) and accuracy (bias) of the yam saponins in the range of 1.79–5.63%

Table 1
RSD of retention time of each yam saponin

Compounds	RSD[a] (%)	
	Retention time	
	Run-to-run	Day-to-day
1	0.39	0.55
2	0.37	0.71
3	0.85	1.52
4	0.78	1.46
5	0.75	1.32
6	0.94	1.89

[a] The result was obtained from 1.0 mg/mL with six measurements and the analytical condition was referred to the text of Section 2.4.

Table 2
Intra- and inter-assay precision (RSD) and accuracy (bias) of yam saponins measurements

Compounds	Nominal concentration (mg/mL)	Intra-assay			Inter-assay		
		Observed concentration (mg/mL)[a]	RSD (%)	Bias (%)	Observed concentration (mg/mL)[a]	RSD (%)	Bias (%)
1	0.20	0.202 ± 0.008	3.96	1.0	0.201 ± 0.012	5.97	0.5
	0.50	0.496 ± 0.018	3.63	−0.8	0.495 ± 0.029	5.86	−1.0
	1.00	0.997 ± 0.029	2.91	−0.3	1.004 ± 0.027	2.69	0.4
2	0.20	0.198 ± 0.010	5.05	−1.0	0.198 ± 0.005	2.53	−1.0
	0.50	0.498 ± 0.009	1.81	−0.4	0.500 ± 0.014	2.80	0.08
	1.00	1.002 ± 0.021	2.10	0.2	1.003 ± 0.035	3.49	0.3
3	0.20	0.203 ± 0.007	3.45	1.5	0.203 ± 0.007	3.45	1.5
	0.50	0.497 ± 0.028	5.63	−0.6	0.497 ± 0.028	5.64	−0.6
	1.00	0.999 ± 0.035	3.50	−0.1	0.999 ± 0.035	3.51	−0.1
4	0.20	0.197 ± 0.010	5.08	−1.5	0.197 ± 0.010	5.08	−1.5
	0.50	0.495 ± 0.024	4.85	−1.0	0.511 ± 0.029	5.68	2.2
	1.00	1.003 ± 0.018	1.79	0.3	0.995 ± 0.026	2.61	−0.5
5	0.20	0.198 ± 0.008	4.04	−1.0	0.202 ± 0.012	5.94	1.0
	0.50	0.498 ± 0.026	5.22	−0.4	0.496 ± 0.042	8.47	−0.8
	1.00	1.004 ± 0.021	2.09	0.4	0.999 ± 0.018	1.80	−0.1
6	0.20	0.202 ± 0.008	3.96	1.0	0.196 ± 0.009	4.59	−2.0
	0.50	0.498 ± 0.018	3.61	−0.4	0.492 ± 0.032	6.50	−1.6
	1.00	0.996 ± 0.031	3.11	−0.4	0.993 ± 0.024	2.42	−0.7

[a] All values are mean ± SD obtained by six replicate analyses.

and −1.5% to 1.5% for intra-assay, and 1.80–8.47% and −2.0% to 2.2% for inter-assay, respectively. Tasi and Liu (2004) indicated that the lowest acceptable reproducibility for accuracy and precision values were ±15%. Therefore, this HPLC method for the yam saponins separation showed good reproducibility.

3.2. Evaluation of SPE for yam saponins

Because the crude extracts of cortex, leaf and vine of yam were stringier and contained a considerable amount of impurities that would hinder the HPLC analysis of the saponins, we modified a previous method (Yang et al., 2003b) and adopted a C18 cartridge to purify steroidal saponins further. The suitable condition to elute the six steroidal saponin standards was estimated in advance. At the first, the mixture of these saponins standards were dissolved in MeOH and diluted with H_2O to turn into 25% MeOH solution. The solution was then loaded onto a C18 cartridge followed by saponins elution. The compound **1** was not eluted until the cartridge with 55% MeOH, compounds **2**–**6** were then eluted in order. All of these saponins were completely eluted from the cartridge with 80% MeOH. For this reason, the condition of SPE of the saponins was described as following: samples (extracts) were dissolved in 1 mL of MeOH and diluted with equal amount of H_2O. After loaded the solution onto a C18 cartridge, the cartridge was washed to remove the polar impurities with 3 mL of water and 3 mL of 50% MeOH (in H_2O), and the saponins were eluted with 3 mL of 80% MeOH (in H_2O) further. The recoveries of the six saponins after extraction with the SPE method were about 99.48–100.08% when the amounts of saponins passing through the C18 cartridge were from 0.2 to 1 mg (Table 3). Hence, we were confident to take this developed method for the yam saponins purification.

3.3. The contents of saponins in various yam organs

The SPE with C18 cartridge is an adequate pretreatment method for yam samples to reduce the interferences from impurities before HPLC analysis of saponins. The recoveries of added saponins in different yam organs were 98.34–99.92% for tuber flesh, 95.98–98.89% for tuber cortex, 97.89–99.44% for rhizophor, 93.82–98.01% for leaf and 93.87–97.65% for vine (Table 4). The recoveries in Table 4 were slightly lower than those in Table 3. This loss of saponins in yam organs might arise from the saponins remained on the tube wall during extraction from these yam samples, which contained viscous materials. Consequently, lower recovery took place while adding lower quantities of saponins to yam samples, especially for leaf and vine. Yang and Hwang (2006) also found the same phenomenon when a C18 SPE method was used for statins extraction from Pu-Erh tea.

Fig. 2 shows the chromatograms of steroidal saponins in various organs of yam. Table 5 exhibits the saponin contents in these organs. The tuber cortex of yam had the highest amount of saponins (582.53 μg/g dw), 389.87 μg/g dw for total furostanol glycosides and 192.66 μg/g dw for total spirostanol glycosides, which was higher than that present in the tuber flesh about 2.55 times (227.86 μg/g dw for total saponins consisted of 128.31 μg/g dw of total furostanol glycosides and 99.55 μg/g dw of total spirostanol glyco-

Table 3
The recoveries of yam saponins after passing through the C18 solid-phase extraction cartridge

Added amount (mg)	% Recovery (%CV)[a,b]					
	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**	Compound **6**
0.20	99.48 (2.62) a	99.75 (4.52) a	99.54 (3.37) a	99.62 (2.84) a	100.01 (5.03) a	99.88 (4.13) a
0.50	99.61 (2.57) a	99.87 (3.75) a	99.65 (1.97) a	99.78 (1.57) a	99.86 (3.54) a	99.78 (3.11) a
1.00	99.53 (3.38) a	100.02 (2.69) a	99.74 (3.08) a	99.89 (2.53) a	100.08 (1.08) a	100.04 (2.79) a

Values bearing different letters in the same column are significantly different ($p < 0.05$).
[a] All values are the means of triplicate analyses.
[b] Values in parentheses are the coefficient of variation (%).

Table 4
The recoveries of added saponins in different organs of yam after extraction

Organ	Added amount (mg)	% Recovery (%CV) [a,b]					
		Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**	Compound **6**
Tuber flesh	0.20	98.78 (4.62) bc	99.08 (4.52) bcd	98.74 (5.30) bc	99.08 (2.84) b	98.91 (4.02) cd	98.34 (4.54) cd
	0.50	99.05 (3.57) ab	99.41 (6.05) b	99.60 (4.54) a	99.52 (4.37) ab	99.53 (3.91) ab	99.42 (5.27) b
	1.00	99.59 (3.38) a	99.83 (5.69) a	99.56 (3.28) a	99.92 (6.21) a	99.72 (2.67) a	99.85 (3.83) a
Tuber cortex	0.20	96.35 (5.27) e	96.08 (6.27) i	97.04 (4.49) e	96.55 (5.61) f	95.98 (2.87) f	96.47 (4.50) hi
	0.50	97.15 (3.41) d	97.49 (5.65) g	97.22 (4.82) e	97.31 (3.60) e	97.38 (7.13) e	96.72 (5.38) gh
	1.00	98.54 (4.53) bc	98.89 (3.81) cde	98.87 (5.69) b	98.34 (7.02) c	98.46 (4.29) d	97.90 (4.23) e
Rhizophor	0.20	98.26 (2.95) c	98.59 (5.06) e	98.13 (5.64) d	97.89 (4.62) cd	98.38 (4.05) d	97.97 (3.96) de
	0.50	98.64 (5.15) bcd	98.82 (4.94) de	98.22 (3.68) cd	98.39 (4.06) c	99.06 (2.66) bc	98.60 (4.89) c
	1.00	99.09 (4.29) ab	99.29 (5.10) bc	98.93 (2.40) b	99.36 (5.27) b	99.24 (4.78) abc	99.39 (5.04) b
Leaf	0.20	94.03 (5.84) f	94.21 (6.70) j	93.82 (7.06) g	94.12 (5.76) h	95.03 (4.68) g	94.32 (6.44) j
	0.50	96.35 (7.12) e	96.63 (3.91) h	95.74 (5.51) f	95.98 (6.18) g	95.64 (5.94) f	96.19 (5.06) i
	1.00	97.48 (3.72) d	98.01 (7.61) f	97.31 (5.37) e	97.66 (4.43) de	97.46 (5.12) e	96.96 (7.21) fg
Vine	0.20	93.92 (8.04) f	94.23 (6.70) j	94.10 (6.86) g	93.87 (6.23) h	94.64 (3.48) g	94.64 (7.21) j
	0.50	96.03 (5.31) e	96.77 (4.52) h	95.79 (7.35) f	96.22 (3.14) gf	95.78 (4.26) f	96.21 (5.72) i
	1.00	97.07 (4.24) d	97.65 (5.79) fg	97.44 (4.92) e	97.57 (5.17) de	97.52 (6.97) e	97.16 (6.15) f

Values bearing different letters in the same column are significantly different ($p < 0.05$).
[a] All values are the means of triplicate analyses.
[b] Values in parentheses are the coefficient of variation (%).

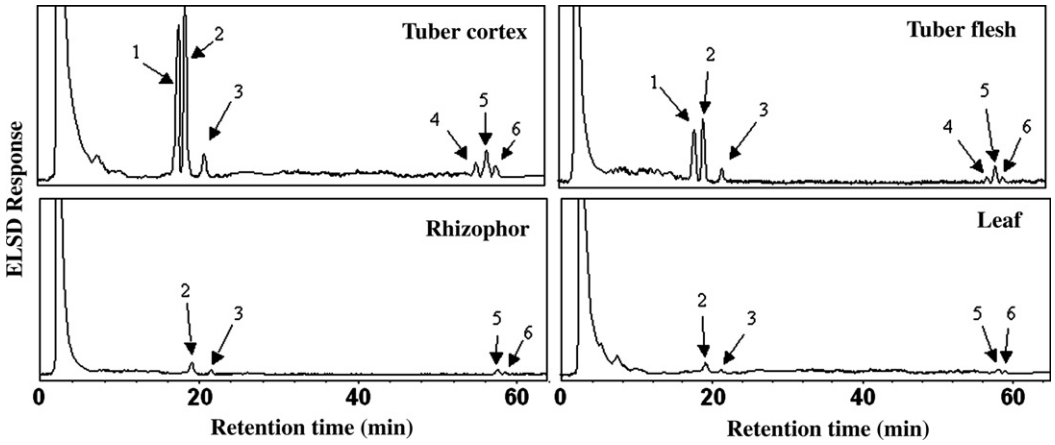

Fig. 2. HPLC chromatograms of saponin extracts of various yam organs. HPLC conditions: column, Luna C18 (4.6 mm i.d. × 250 mm, 5 μm); mobile phase, MeOH/H_2O = 62/38 (v/v) from 0 to 20 min and 71/29 (v/v) from 21 to 65 min; flow rate, 1 mL/min; detection, evaporative light scattering detector (ELSD) (tube temperature, 75 °C; gas flow rate, 2.8 mL/min).

sides). Compounds **1** and **4** could not be detected in the rhizophor, leaf and vine of yam. There was 29.39 μg/g dw of saponins (13.37 μg/g dw for total furostanol glycosides and 16.02 μg/g dw of total siprostanol glycosides) in yam rhizophor. The lowest amounts of saponins appeared in yam leaf (24.41 μg/g dw for total saponins consisted

Table 5
Saponin contents in varied organs of yam

Compounds	Saponin contents (μg/g dw)[a]				
	Tuber flesh	Tuber cortex	Rhizophor	Leaf	Vine
1	48.24 ± 3.21 b	149.63 ± 8.37 a	ND[b]	ND	ND
2	50.13 ± 2.37 b	156.42 ± 9.12 a	7.13 ± 2.07 c	5.12 ± 1.04 d	4.98 ± 1.15 d
3	29.94 ± 2.17 b	83.82 ± 5.21 a	6.24 ± 0.77 c	7.03 ± 1.23 c	7.15 ± 2.04 c
Total furostanol glycosides	128.31 ± 7.75 b	389.87 ± 22.70 a	13.37 ± 2.84 c	12.15 ± 2.27 d	12.13 ± 3.19 d
4	32.93 ± 2.54 b	70.32 ± 4.64 a	ND	ND	ND
5	45.24 ± 1.83 b	81.95 ± 2.97 a	10.64 ± 2.08 c	6.02 ± 1.13 d	6.21 ± 1.24 d
6	21.38 ± 3.04 b	40.39 ± 3.04 a	5.38 ± 0.92 d	6.24 ± 1.31 c	5.62 ± 0.95 d
Total spirostanol glycosides	99.55 ± 7.41 b	192.66 ± 7.41 a	16.02 ± 3.00 c	12.26 ± 2.44 d	11.83 ± 2.19 d
Total saponins	227.86 ± 15.16 b	582.53 ± 15.16 a	29.39 ± 5.84 c	24.41 ± 4.71 d	23.96 ± 3.38 d

Values bearing different letters in the same row are significantly different ($p < 0.05$).
[a] All values are mean ± SD obtained by triplicate analyses.
[b] ND = not detected.

of 12.15 μg/g dw of total furostanol glycosides and 12.26 μg/g dw of total spirostanol glycosides) and vine (23.96 μg/g dw for total saponins consisted of 12.13 μg/g dw of total furostanol glycosides and 11.83 μg/g dw of total spirostanol glycosides). The results could support the report of Dinan et al. (2001). They described that notable variation of saponin quantity could be found in plant organs. In a previous study, Yang et al. (2003b) determined saponins in the tuber flesh of yam (*D. pseudojaponica* Yamamoto) merely and found their total amounts were 216.66 μg/g dw.

We expect the results of the study could enhance the exploitation of yam plant, especially for yam tuber cortex, which contains abundant steroidal saponins compared to other organs.

Acknowledgments

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