Qualitative and Quantitative Analysis of Swertia Herbs by High Performance Liquid Chromatography-Diode Array Detector-Mass Spectrometry (HPLC-DAD-MS)

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We evaluated the composition of Swertia herbs using high performance liquid chromatography-diode array detector-mass spectrometry (HPLC-DAD-MS). Eleven peaks of 6 species were unequivocally identified by comparing their retention times, UV spectra, on-line electrospray ionization mass (ESI-MS) spectra, and collision-induced dissociation mass spectrometry/mass spectrometry (CID-MS/MS) data with those of authentic compounds. We adopted wavelengths of 254 nm, 340 nm and 230 nm to simultaneously determine these 11 compounds. By comparing the overall DAD and total ion current (TIC) profiles of various samples, the 6 species were differentiated in terms of the occurrence and/or relative concentrations of the eleven compounds. Our novel validated HPLC-DAD-MS method not only facilitates quality control and identification of Swertia herbs, but is also applicable to systematic investigations of the distribution of secoiridoids, flavonoids, and xanthones in the genus Swertia.

Key words Swertia herb; liquid chromatography-mass spectrometry; quality control; quantitative determination

Swertia herbs belonging to Gentianaceae have been used to treat digestive dysfunction, acute and chronic dysentery, and canker in China, Japan and Korea and to treat hepatitis.[1] They might also promote liver regeneration, repair liver fibrillation, clear fatty deposits within the liver and protect the liver from experimental damage by CCl_4.[2]

Swertia herbs contain the major secondary metabolites iridoid glucosides, xanthones and flavonoids.[3—8] Of these, swertiamarin (**1**) is the most abundant active constituent, being a central nervous system-depressant[9] and anticholinergic.[10] Sweroside (**2**) inhibits the growth of *Staphylococcus epidermidis*. Both swertiamarin (**1**) and sweroside (**2**) inhibit the growth of *Bacillus cereus*, *Bacillus subtilis* and *Citrobacter freundii*, are generally toxic in the brine shrimp lethality bioassay,[11] and have mild hepatoprotective activity.[12] Amarogentin (**4**) is the most bitter principle and it has antiproliferative and pro-apoptotic actions.[13] Swertianolin (**6**) has anti-acetylcholinesterase activity.[14] Bellidifolin (**5**) has potent and dose-dependent hypoglycemic activity in rats with streptozotocin (STZ)-induced diabetes.[15] Pseudonolin (**7**) exhibits strong hepatoprotective activity against cultured hepatocytes injured by CCl_4.[1] Isoorientin (**8**) has powerful antiperoxidative activity towards linoleic acid and protects against the bactericidal action of the *tert*-butyl peroxyl radical.[16] Isoorientin (**8**) also has significant anti-nociceptive and anti-inflammatory activities.[17] Isovitexin (**9**) is a potent antioxidant that reduces hydrogen peroxide formation in mice.[18]

The content of each component in Swertia herbs significantly varies according to geographic, climatic, environmental and other factors. As the application of Swertia herbs becomes more extensive, a quality standard is urgently required to identify the raw materials. Quality control and evaluation of Swertia herbs have generally targeted swertiamarin (**1**), due to the high content of this compound.[19,20] The content of swertiamarin (**1**)[21] and its seasonal variation have been determined using thin-layer chromatography.[22] Furthermore, swertiamarin (**1**) has been quantified using high-performance liquid chromatography (HPLC).[23,24] However, this compound alone could not be responsible for the overall pharmacological actions of Swertia herbs and synergistic effects among the various constituents probably play significant roles. The fingerprints of Swertia herbs or multi-component chromatograms using HPLC or capillary electrophoresis have been published,[24,25] but we describe here the qualitative and quantitative analysis of 6 Swertia herbs using HPLC-diode array detection (DAD)-MS as well as the chemical evaluation of these species by comparing the overall DAD and total ion current (TIC) profiles.

We identified characteristic peaks among various samples and determined the optimal wavelength at which to detect each constituent characteristic chromatograms using HPLC-DAD, in particular the analysis of three-dimensional retention time-absorbance-wavelength plots (3D-plots). The chemical constituents in the chromatographic profiles were identified based on findings of on-line HPLC-electron spray ionization (ESI)-MS. The powerful HPLC-ESI-MS technique is highly sensitive and selective and thus is widely applied to analyze complex mixtures. By coupling HPLC to an ion trap mass spectrometer, valuable structural information can be obtained from collision-induced dissociation (CID). In this study, we used HPLC-ESI-MS to confirm the structures of the main constituents of Swertia herbs and showed that this method is useful for their quality control.

Experimental

Instrumentation Chromatograms, UV spectra and 3D-plots were acquired using an Agilent 1100 series HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) comprising a binary pump, thermostated col-

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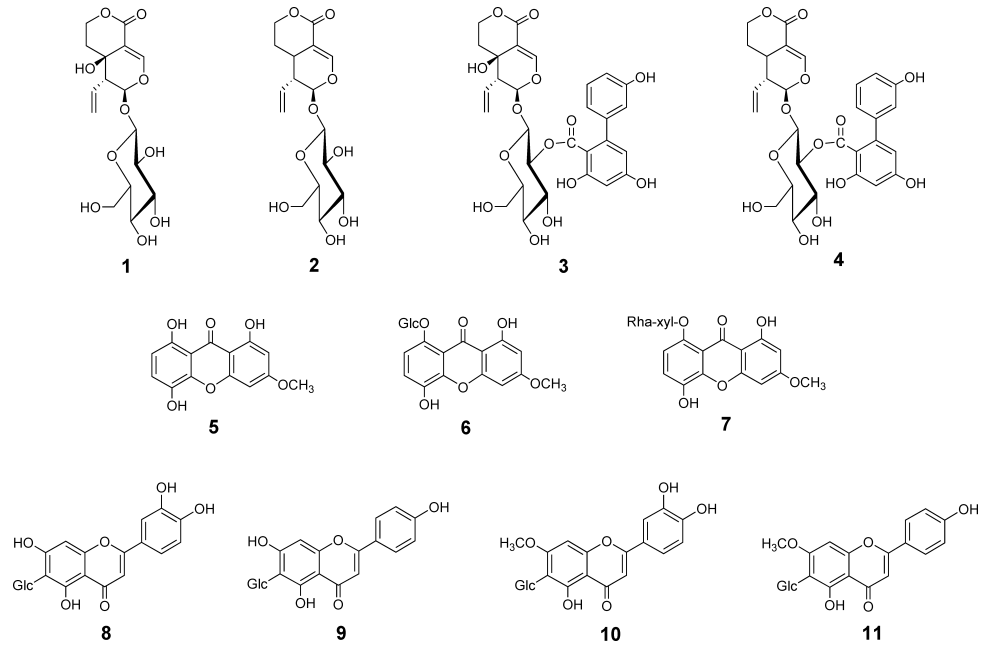

Fig. 1. Chemical Structures of Swertia Components

Swertiamarin (**1**), sweroside (**2**), amaroswerin (**3**), amarogentin (**4**), bellidifolin (**5**), swertianolin (**6**), pseudonolin (**7**), isoorientin (**8**), isovitexin (**9**), swertiajaponin (**10**) and swertisin (**11**).

umn compartment and DAD. We used an Esquire 3000 mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) system with an ESI ionization source for mass spectrometric identification of characteristic peaks. Samples were separated by HPLC using a TSK gel ODS-80 Ts (2.0 mm×150 mm i.d., 5 μm) column with a flow rate of 1.0 ml/min at 30 °C. Solvent systems were applied in a stepwise gradient of acetonitrile–water starting from 10% (v/v) acetonitrile followed by 0—15 min, 10—35% acetonitrile; 15—20 min, 35—100% acetonitrile. Thereafter, the column was washed with 100% acetonitrile for 10 min and equilibrated with the starting eluant for 10 min. The ESI-MS conditions comprised drying gas N_2, 10.0 l/min; temperature, 250 °C; nebulizer pressure, 50 psi; isolation width, 4; fragment amplification, 1.0; scan range, 50—750 u.

Solvents and Chemicals Acetonitrile and methanol of analytical HPLC grade as well as chloroform were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Reference Compounds Standard compounds (**1**—**11**, Fig. 1) were isolated from a MeOH extract obtained from *Swertia japonica* as follows. Dried whole plants of *S. japonica* (5 kg) were extracted with MeOH (25 l) three times at room temperature, and the extract (998 g) was dried under reduced pressure, suspended in water (1.2 l) and extracted three times with $CHCl_3$ (1.2 l). The aqueous layer was passed through a Mitsubishi Diaion HP-20 column (6×150 cm) and eluted with H_2O, 50%, and 100% MeOH. The 50% MeOH fraction (10 g) was separated by chromatography on a silica-gel column (5×60 cm) using $CHCl_3$–MeOH as the eluent to yield swertiamarin (**1**, 7.8 g) and sweroside (**2**, 20 mg). The MeOH eluate (140 g) was separated by silica-gel column chromatography (8×100 cm) using $CHCl_3$–MeOH to yield amarogentin (**4**, 0.20 g), amaroswerin (**3**, 0.45 g), bellidifolin (**5**, 1.28 g), swertianolin (**6**, 1.17 g), pseudonolin (**7**, 0.43 g), isoorientin (**8**, 2.65 g), isovitexin (**9**, 0.16 g), swertiajaponin (**10**, 1.57 g) and swertisin (**11**, 0.74 g).

The structures of these compounds were identified by comparing their ^{1}H- and ^{13}C-NMR, MS (ESI), and UV spectral data with those reported,[1,3—7,26—28] and the purities were checked by HPLC.

Plant Materials *Swertia japonica* Makino (*S. japonica* I, cultivated in 2004 at Nagano, Japan; *S. japonica* II, cultivated in 2005 at Nagano, Japan; *S. japonica* III, cultivated in 2006 at Gifu, Japan) and *Swertia pseudochinensis* Hara (collected in Hebei province, China) were supplied by ALPS Pharmaceutical Ind. Co., Ltd., Japan, *Swertia decora* Franch, *Swertia delavayi* Franch, *Swertia binchuangensis* Franch and *Swertia punicea* Hemsl were collected in 2004 from Yunnan province, China, and identified by Prof. Tingnong He (Northwest Institute of Plateau Biology, Chinese Academy of Science). Voucher specimens have been deposited in Museum of Materia Medica, Toyama University.

Sample Preparation Samples of powdered Swertia herbs (1.0 g) were placed in 50 ml amber vials with extracted twice over a period of 1 h each with 15 ml of methanol. The extracts were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml of methanol, passed through a 0.45 μm membrane filter, and then (10 μl) analyzed by HPLC.

Results and Discussion

Optimization of Chromatographic and MS Conditions The constituents of Swertia herbs in published fingerprint and multi-components analyses were detected at wavelengths of 260 and 254 nm.[24,25] The optimal wavelength for detecting Swertia components was determined using a DAD detector. Since peak maximal number, relatively higher peaks and a stable baseline in chromatograms were obtained at 254 nm, we monitored elution profiles at this wavelength.

Active principles from Swertia herbs have been extracted using various solvents including methanol and ethanol.[24,25] However, we used methanol because the extraction efficiency was better than that of ethanol.

Eleven isolated reference compounds were analyzed to optimize the MS measurement conditions. The sensitivity of detecting all of the reference compounds in the ESI mode was higher using negative, than positive ionization. In positive ion mode, amaroswerin (**3**) yielded a weak sodium adduct ion peak at *m*/*z* 603, but a protonated or potassium adduct was not identified. Because the $[M-H]^-$ signals of most compounds were obvious in the negative ion mode (Table 1), all mass spectral data discussed here were obtained in the negative ESI mode.

Identification of Chemical Constituents of Swertia Herbs Eleven peaks in the HPLC/DAD and HPLC/MS (TIC) chromatograms were unequivocally identified through comparisons of their retention times, on-line ESI-MS, CID-MS/MS and UV spectra with those of authentic compounds. Table 1 shows the retention times, ESI-MS, CID-MS/MS and

UV data.

A sugar moiety was consequently eliminated and intense aglycone ions always appeared as a base peak in the CID-MS/MS spectra of *O*-glucosyl xanthones. The aglycone moiety exhibited fragment ions corresponding to the loss of CO and CH_3.

All *C*-glycosidic flavonoids showed intense $[M-H]^-$ ions in negative ESI-MS spectra. Contrary to *O*-glycosyl xanthones, *C*-glycosyl flavonoids did not generate abundant aglycone ions, but produced characteristic ions owing to the fragmentation of the *C*-glycosyl group caused by the loss of 2(HCHO), 3(HCHO) and 4(HCHO).[29]

Various characteristic fragmentations for iridoids (**1**—**4**) were generated with the elimination of neutral molecules from either the aglycone or sugar moieties, cleavage of the aglycone or sugar moiety, and elimination of the substituents present in the sugar moiety.

Suryawanshi *et al.*[30] have reported the fragmentation processes of *C*-glycosidic xanthone and secoiridoids in a positive ion mode with a triple quadrupole mass spectrometer, but we found that a negative ion mode that we adopted was more suitable for identification of secoirioids in Swertia herbs with an ion trap mass spectrometer and there were some differences in fragmentation between the two modes.

Quantitation of Chemical Components in Swertia Herbs Although some of the main components in *S. japonica* have been quantified at a wavelength of 254 nm,[24] components at their respective maximum absorption wavelengths have not yet been simultaneously determined. The absorption maxima of the isolated xanthones, flavonoids and secoiridoids were 254, 340 and 230 nm, respectively (Fig. 2), and were thus quantified at these wavelengths. Although belonging to a secoiridoid group, sweroside (**2**) was quantified at 254 nm.

A stock solution of the 11 standard compounds including 4 secoiridoids, 4 flavonoids and 3 xanthones was prepared in

Table 1. Chromatographic and Spectrometric Data of 11 Swertia Herbal Components

Peak No.	t_R (min)	$[M+H]^+$ (*m*/*z*)	$[M-H]^-$ (*m*/*z*)	Other negative ions (*m*/*z*)	λ_{max} (nm)	Component
1	6.64	375	—	747 $[2M-H]^-$, 409, 411 $[M+Cl]^-$, 179 $[Glc-H]^-$	238	Swertiamarin (**1**)
2	7.94	359	357	715 $[2M-H]^-$, 195 $[M-H-Glc]^-$, 167 $[M-H-Glc-C_2H_4]^-$, 125	245	Sweroside (**2**)
3	8.99	449	447	429 $[M-H-H_2O]^-$, 411 $[M-H-H_2O]^-$, 357 $[M-H-3(HCHO)]^-$, 327 $[M-H-4(HCHO)]^-$	210, 269, 348	Isoorientin (**8**)
4	9.26	463	461	371 $[M-H-HCHO]^-$, 341 $[M-H-2(HCHO)]^-$, 313 $[M-H-2(HCHO)-CO]^-$, 298 $[M-H-2(HCHO)-CO-H_2O]^-$	210, 270, 348	Swertiajaponin (**10**)
5	10.58	433	431	413 $[M-H-H_2O]^-$, 341 $[M-H-3(HCHO)]^-$, 311 $[M-H-4(HCHO)]^-$	213, 272, 340	Isovitexin (**9**)
6	10.99	447	445	385 $[M-H-2(HCHO)]^-$, 355 $[M-H-3(HCHO)]^-$, 325 $[M-H-4(HCHO)]^-$, 297 $[M-H-4(HCHO)-CO]^-$	210, 270, 341	Swertisin (**11**)
7	13.43	437	435	315 $[M-H-4(HCHO)]^-$, 297 $[M-H-4(HCHO)-H_2O]^-$, 273 $[Aglycone-H]^-$	258, 275, 325	Swertianolin (**6**)
8	14.00	—	601	637, 639 $[M+Cl]^-$, 583 $[M-H-H_2O]^-$, 539 $[M-H-H_2O-CO_2]^-$, 407 $[M-H-Aglycone]^-$, 347 $[M-H-Aglycone-2(HCHO)]^-$, 317 $[M-H-Aglycone-3(HCHO)]^-$, 287 $[M-H-Aglycone-4(HCHO)]^-$, 245 $[M-H-Aglycone-Glc]^-$, 227 $[M-H-Aglycone-Glc-H_2O]^-$	225, 270, 310	Amaroswerin (**3**)
9	14.86	587	585	621, 623 $[M+Cl]^-$, 245 $[M-H-Aglycone-Glc]^-$, 227 $[M-H-Aglycone-Glc-H_2O]^-$	225, 270, 310	Amarogentin (**4**)
10	17.97	553	551	405 $[M-H-Rha]^-$, 387 $[M-H-Rha-H_2O]^-$, 273 $[M-H-Rha-Xyl]^-$	258, 330	Pseudonolin (**7**)
11	20.36	275	273	258 $[M-H-CH_3]^-$, 230 $[M-H-CH_3-CO]^-$	250, 280, 335	Bellidifolin (**5**)

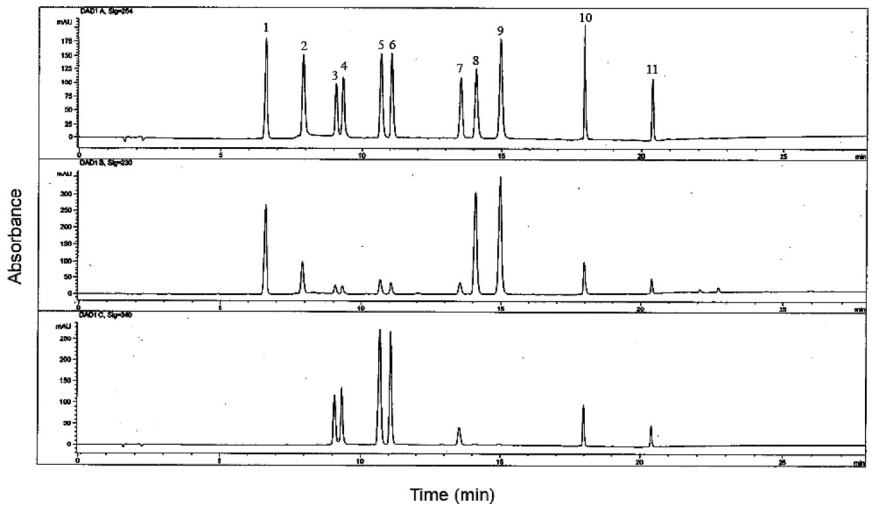

Fig. 2. HPLC Elution Profiles Monitored at 254 (Xanthones), 230 (Secoiridoids) and 340 nm (Flavonoids)

Peaks: 1, swertiamarin (**1**); 2, sweroside (**2**); 3, isoorientin (**8**); 4, swertiajaponin (**10**); 5, isovitexin (**9**); 6, swertisin (**11**); 7, swertianolin (**6**); 8, amaroswerin (**3**); 9, amarogentin (**4**); 10, pseudonolin (**7**); 11, bellidifolin (**5**).

Table 2. Linear Regression Data and Validation of Method for Identifying Swertia Herbal Components

Standard	Regression equation	*R*	Linear range (μg/μl)	Recovery (%)		Intraday (%)	Interday (%)	LOD (pg/μl)	LOQ (pg/μl)
				Mean	RSD	RSD	RSD		
Swertiamarin (**1**)	Y=4968.91X+1.28	0.9995	0.1017—4.0664	97.3	3.0	3.1	5.3	254	1017
Sweroside (**2**)	Y=8550.05X+3.30	0.9997	0.0011—0.1047	101.3	4.2	3.2	4.1	314	1047
Isoorientin (**8**)	Y=7682.38X+1.38	0.9995	0.0100—0.4975	99.5	3.2	4.0	6.4	498	995
Swertiajaponin (**10**)	Y=8752.05X+0.56	0.9995	0.0010—0.2510	97.3	3.8	2.8	3.5	201	1004
Isovitexin (**9**)	Y=11085.55X+0.26	0.9991	0.0005—0.2056	97.8	3.5	3.2	3.6	206	514
Swertisin (**11**)	Y=9173.16X+20.86	0.9994	0.0003—1.0120	96.4	3.0	2.9	5.1	81	304
Swertianolin (**6**)	Y=16685.91X+8.50	0.9993	0.0003—0.5230	95.5	4.4	4.2	4.7	105	314
Amaroswerin (**3**)	Y=12442.57X+0.94	0.9993	0.0005—0.5000	96.5	4.1	3.0	5.9	150	500
Amarogentin (**4**)	Y=10661.86X+0.81	0.9996	0.0010—0.5100	97.6	3.3	2.8	3.9	200	1000
Pseudonolin (**7**)	Y=12315.71X+2.13	0.9991	0.0003—0.1617	98.3	2.9	3.3	6.1	108	323
Bellidifolin (**5**)	Y=7792.78X+1.76	0.9995	0.0010—1.0060	98.6	2.7	3.2	5.0	302	1006

R, correlation coefficients for standard curves; RSD, ratio of relative standard deviation; LOD, limit of detection; LOQ, limit of quantitation.

Table 3. Contents (μg/g Herb) of Components of Whole Swertia Herbs from Various Localities

Locality	*S. japonica* I	*S. japonica* II	*S. japonica* III	*S. pseudochinensis*	*S. delavayi*	*S. decora*	*S. binchuangensis*	*S. punicea*
Swertiamarin (**1**)	40538.1±1928.4	40569.6±957.1	38511.4±1060.8	10831.6±652.6	29367.0±1566.5	37238.5±793.2	6378.1±238.4	1368.6±85.6
Sweroside (**2**)	496.4±13.8	720.0±26.2	531.8±9.4	308.3±7.8	196.0±6.6	421.2±15.1	nd	392.6±8.3
Isoorientin (**8**)	4856.8±167.2	4704.3±221.5	3835.5±204.9	796.5±18.7	711.0±35.1	1785.8±85.2	180.0±7.5	144.2±5.7
Swertiajaponin (**10**)	2191.2±110.6	1258.5±50.5	943.4±33.0	122.7±5.2	nd	nd	nd	nd
Isovitexin (**9**)	684.2±27.1	1158.1±52.7	1635.0±76.2	362.1±9.2	1511.2±50.6	991.7±48.3	nd	nd
Swertisin (**11**)	5291.7±313.5	7457.2±455.8	8225.7±416.2	2510.8±93.5	nd	nd	66.7±1.4	nd
Swertianolin (**6**)	2594.8±95.2	5856.7±268.5	1052.6±42.1	380.3±10.7	nd	nd	1034.7±44.8	301.0±7.6
Amaroswerin (**3**)	1279.8±45.9	4534.7±224.6	2935.0±143.6	nd	nd	nd	602.6±21.2	nd
Amarogentin (**4**)	904.3±36.4	3435.4±146.7	2879.0±88.9	nd	nd	nd	242.1±7.4	169.9±5.5
Pseudonolin (**7**)	1201.3±53.7	934.8±39.3	1087.7±51.2	103.7±2.2	nd	nd	116.3±3.5	129.9±6.4
Bellidifolin (**5**)	4229.2±134.6	8569.6±331.4	7886.5±420.5	734.2±30.8	nd	nd	178.1±5.2	485.1±11.3

The value is mean±S.D. (n=3). nd: not detected.

methanol. The stock solution was serially diluted to reach the appropriate concentration ranges for the construction of calibration curves. The diluted standards were filtered through a 0.45 μm membrane and then analyzed by HPLC. Calibration curves were constructed for each compound at 6 concentrations in triplicate by plotting the peak area (Y) *versus* concentration (X). Linear regression of the standard compounds showed good linearity, and the method permitted the determination of these constituents in Swertia herbs over a wide range of concentrations.

To verify the precision of the method, we analyzed standard mixtures 5 times on the same day (intraday) and on 3 consecutive days (interday). The relative standard deviations (RSDs) of the intraday and interday are as shown in Table 2.

Samples of *S. japonica* I (n=3) were spiked with a known amount of the eleven isolated compounds to examine recovery and RSD values using the proposed method (Table 2). The limit of detection (LOD) and the limit of quantitation (LOQ) under our chromatographic conditions were determined by measuring the magnitude of the analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting a series of solutions until the S/N ratio reached 3 for LOD and 10 for LOQ. Five replicate injections of the solution resulted in an RSD of 3%. Table 2 shows the LOD and LOQ for each compound.

The amounts of the 11 analytes in Swertia herbs were determined within a linear range by comparison with the calibration curve of each standard compound (Table 3).

Comparison of Swertia Herbs The components of Swertia herbs considerably differ according to their source. Various species of Swertia herbs have historically been identified by sophisticated botanists based on morphological and histological characteristics, but this is time consuming and not appropriate for many researchers. Therefore, a simple and reliable technique should be established to unambiguously distinguish the various species.

The sensitivity of UV monitoring is very low and structural information is limited. This problem can be overcome by using an on line LC-MS method. By comparing the overall DAD and TIC profiles of various samples, we could discriminate six species in terms of the occurrence and/or relative concentration of 11 compounds.

Figures 3 and 4 show typical DAD and TIC chromatograms of the extracts of 6 species of Swertia herbs. Agreement between the TIC and UV chromatograms was close. Notably, many compounds such as amaroswerin (**3**) and amarogentin (**4**) generate very small peaks on UV chromatograms that can easily be overlooked, whereas TIC clearly identified the two components. On the contrary, peaks of bellidifolin (**5**) and swertianolin (**6**) were relatively weak in the TIC, possibly because of poor ionization efficiency.

Swertiamarin (**1**) was the most dominant in all of the Swertia samples, the contents varying from about 1400 to 40600 μg/g in 6 species, a variation of about 30-fold. Although amaroswerin (**3**) and amarogentin (**4**) have been isolated from *S. pseudochinensis*,[1)] their contents were below the limit of detection in the present study. On the other hand,

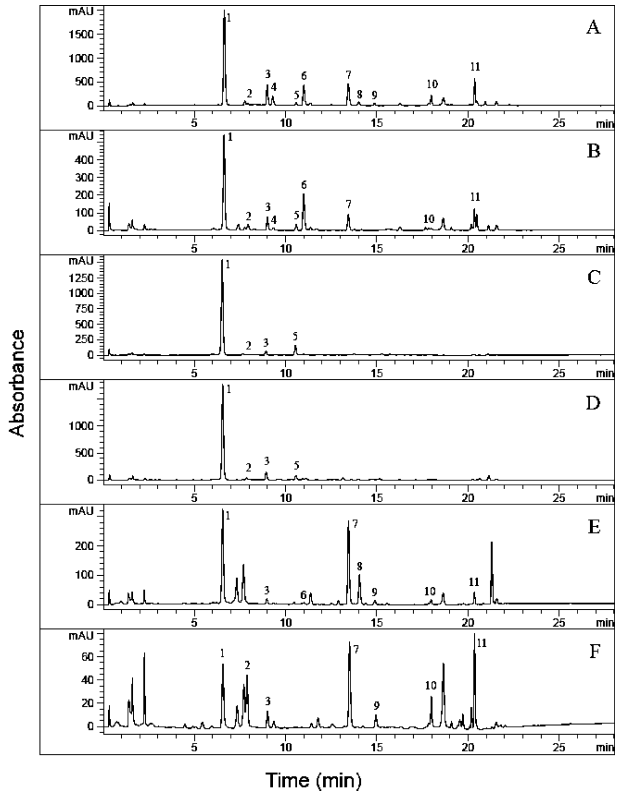

Fig. 3. Comparison of HPLC-DAD Chromatograms (254 nm) of Swertia Herbs

A, *S. japonica*; B, *S. pseudochinensis*; C, *S. delavayi*; D, *S. decora*; E, *S. binchuangensis*; F, *S. punicea*.

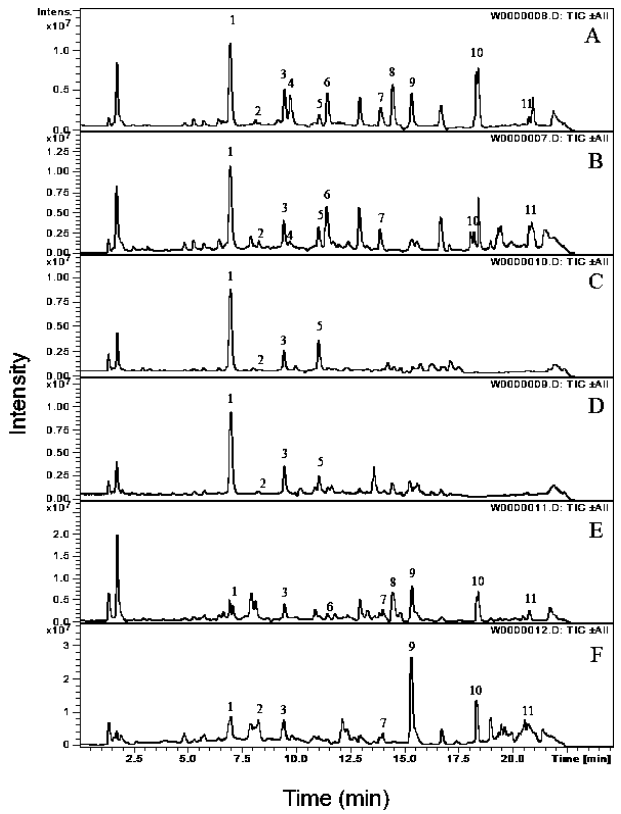

Fig. 4. Comparison of Total Ion Chromatograms (TIC) of Swertia Herbs

A, *S. japonica*; B, *S. pseudochinensis*; C, *S. delavayi*; D, *S. decora*; E, *S. binchuangensis*; F, *S. punicea*.

significant amounts were found in *S. japonica*, and they were also detected in *S. binchuangensis* and *S. punicea*. Amarogentin (**4**) has a bitterness index of 58000000 compared with that of quinine, at 200000.[31] The absence of amaroswerin (**3**) and amarogentin (**4**) in *S. pseudochinensis* might explain why this herb is less bitter than *S. japonica* as recorded in the Instruction Manual of the Japanese Pharmacopoeia (14th edition).[32]

Pseudonolin (**7**) has been isolated from *S. pseudochinensis*,[1] but we also isolated it from *S. japonica*, and discovered it here in *S. binchuangensis* and *S. punicea*. *S. japonica* contained about 10-fold more pseudonolin (**7**) than *S. pseudochinensis*. Bellidifolin (**5**) and swertianolin (**6**) were abundant in *S. japonica*, present in *S. pseudochinensis*, *S. binchuangensis*, *S. punicea*, but absent in *S. decora* and *S. delavayi*.

The relative amounts of *C*-glycosidic flavonoids are in the order of swertisin (**11**)>isoorientin (**8**)>swertiajaponin (**10**)>isovitexin (**9**) in *S. japonica* according to paper partition chromatography (PPC). The present results are not consistent with these findings. We assume that this is mainly due to the difference in accuracy of detection method. Swertiajaponin (**10**) is abundant in *S. japonica* and present in *S. pseudochinensis*, but undetectable in other species and can thus be regarded as a characteristic marker of these two species.

The DAD and TIC chromatograms obtained using our established techniques provided the most comprehensive chemical information for the identification and assessment of Swertia herbs. We concluded that the content and composition of components significantly differs among various Swertia species. Our novel, validated HPLC-DAD-MS method will not only facilitate the quality control and identification of Swertia herbs, but could also advance systematic investigation of the distribution of secoiridoids, flavonoids and xanthones in the genus Swertia to maximize the resources of these and other medicinal plants.

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