Phytosterols in Sea Buckthorn (*Hippophaë rhamnoides* L.) Berries: Identification and Effects of Different Origins and Harvesting Times

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Sterols in seeds, pulp/peel fractions, and whole berries of sea buckthorn (*Hippophaë rhamnoides* L.) samples belonging to two major subspecies (*sinensis* and *rhamnoides*) from Finland and China were analyzed as TMS derivatives by gas chromatography−mass spectrometry after saponification of the oils. The total sterol contents in the seeds, the fresh pulp/peel, and the whole berries were 1200−1800, 240−400, and 340−520 mg/kg, respectively. The corresponding values in the extracted oils were 12−23, 10−29, and 13−33 g/kg. Sitosterol constituted 57−76 and 61−83%, respectively, of the seed and pulp/peel sterols. The sterol content and composition showed little variation between subspecies and collection sites. Different harvesting dates showed significant effects on the levels of some sterols both in the seeds and in the pulp/peel. The sterol profiles obtained are useful for characterizing sea buckthorn and detecting adulterations of the valuable oils. The information provided by the present investigation is also important for further chemical investigation of sea buckthorn sterols and industrial utilization of the berries as a raw material of functional foods.

Keywords: *Berries; sea; buckthorn; harvesting time; seeds; sterols; Hippophaë rhamnoides*

INTRODUCTION

Sea buckthorn (*Hippophaë rhamnoides* L.) is a hardy bush with nutritious berries, naturally distributed mainly in Asia and Europe and also cultivated in North and South America (*1*, *2*). During the past decade, the species has attracted intense global attention not only because of its role in soil and water conservation and reforestation of eroded areas but also because of the nutritional and medicinal values of its berries. The yellow-red, pearl-shaped berries are important sources of vitamins for the local people at the natural growth sites of the plant. The berries have a long history of application in Tibetan and Mongolian medicines and are also an ingredient for traditional Chinese medicines found in the Chinese Pharmacopoeia. A wide spectrum of physiological effects of the berries and berry products has been shown by many animal experiments and the long history of clinical and nutritional practice, especially in China and Russia (*3*, *4*). These positive effects include lowering plasma cholesterol level, inhibiting platelet aggregation, regulating immune function, promoting the repair of injuries on skin and mucosa, and anticancer effects (*5*−*12*).

Recently, interest in sea buckthorn has also developed in Europe, Japan, Canada, and the United States as more information on the chemical composition and physiological effects of the berries has become available. In association with research on sea buckthorn, the industrial cultivation and utilization of its berries as a raw material for functional food and natural medicines have started in these regions.

Although many components, such as vitamin C, sugars, acids, and fatty acids, of the berrries of sea buckthorn have been extensively investigated, very little is known about their sterols. In the few existing publications, only single samples were analyzed, and completely different sterol profiles have been reported (*13*−*19*). A lack of original mass spectrometric data in most of these publications makes an objective evaluation of their identifications impossible. Thus, there is an urgent need for further investigation of sterols in sea buckthorn.

In the present study, we analyzed the seeds, soft parts, and whole berries of two major subspecies of sea buckthorn, sp. *rhamnoides* in Finland and sp. *sinensis* in China, to obtain a general profile of sterols in sea buckthorn berries on the basis of a larger number of samples. This profile will serve as a basis for further detailed chemical investigation and nutritional evaluation of the berries. The aim of the present study was also to compare the difference between the two subspecies and to follow changes in the contents and compositions of sterols in the seeds, soft parts, and whole berries during the harvesting period.

MATERIALS AND METHODS

Berries. Wild berries of *H. rhamnoides* ssp. *sinensis* were picked in six different natural growth sites in China around mid-October 1997. The growth areas ranged between longitudes of 104° 40′ E and 114° 41′ E, latitudes of 35° 06′ N and 40° 03′ N, and altitudes of 1370 and 2800 m. Four samples of wild ssp. *rhamnoides* berries were picked on the shores of the Gulf of Bothnia in southwestern Finland in September 1999. The areas ranged between longitudes of 21° 04′ E and 24° 24 ′E, latitudes of 60° 45′ N and 64° 47′ N, and altitudes of 0 and 50 m. To follow the changes during the harvesting period, wild berries of subsp. *sinensis* were collected at their natural growth sites (Wenshui and Xixian, Shanxi Province, People's Republic of China) from the end of August to the end of November 1998. All samples were loosely frozen in sealed plastic bags immediately after collection to avoid desiccation and extra moisture condensation and stored at −20 °C until analyzed.

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Table 1. Samples of Sea Buckthorn Berries from Different Subspecies and Collecting Times

sample	location	collection date	subspecies	seed content (% of fresh wt)
	Finland			
r1	Siikajoki	Sept 2, 1999	*rhamnoides*	5.7
r2	Vaasa	Sept 3, 1999	*rhamnoides*	4.3
r3	Pyhämaa	Sept 8., 1999	*rhamnoides*	5.6
r4	Pyhämaa	Sept 8, 1999	*rhamnoides*	5.1
	China			
s1	Xunyi, Shaanxi	Oct 12, 1997	*sinensis*	11.5
s2	Youyu, Shanxi	Oct 15, 1997	*sinensis*	7.7
s3	Datong, Qinghai	Oct 13, 1997	*sinensis*	7.6
s4	Dingxi, Gansu	Oct 17, 1997	*sinensis*	9.4
s5	Wenshui, Shanxi	Aug 30, 1998	*sinensis*	5.9
		Sept 15, 1998		6.0
		Sept 30, 1998		5.0
		Oct 15, 19 98		3.6
		Oct 30, 1998		6.1
		Nov 15, 1998		6.7
		Nov 30, 1998		8.4
s6	Xixian, Shanxi	Aug 30, 1998	*sinensis*	5.9
		Sept 15, 1998		5.8
		Sept 30, 1998		4.7
		Oct 15, 1998		4.7
		Oct 15, 1998		4.4
		Nov 15, 1998		3.9
		Nov 30, 1998		3.9

Detailed information of the samples is presented in Table 1.

Lipid Extraction. Berries (400 g) of each sample were taken from a 5 kg lot using a sample partitioning procedure. Seeds were isolated from frozen berries by pressing the juice and rinsing the residue with distilled water. The press residue was air-dried at room temperature, and seeds were separated mechanically. For the whole berry analysis frozen berries were freeze-dried (Dura-Top bulk tray dryer, FTS System Inc., Stone Ridge, NY) to 15−30% of the original weight, depending on the berry composition.

Samples (5 g) of seeds, freeze-dried whole berries, and pulp/peel (seedless parts of the berries) were crushed in a mortar in liquid nitrogen and the lipids isolated using a methanol/chloroform extraction procedure (*20*, *21*). The sample was homogenized in methanol (50 mL) for 1 min in a blender, chloroform (100 mL) was added, and homogenization was continued for a further 2 min. The mixture was filtered and the solid residue resuspended in chloroform/methanol (2:1, v/v, 150 mL) and homogenized for 3 min. The mixture was filtered again and washed with fresh solvent (chloroform/methanol, 2:1, v/v, 150 mL). The combined filtrates were cleaned with a repeat procedure using a 0.88% potassium chloride water solution followed by a methanol/water (1:1, v/v) solution. The purified lipids were filtered before the solvent was removed on a rotary evaporator.

Saponification of Lipids. After the addition of cholesterol palmitate (1.6 mg) as an internal standard, lipids (400 mg) were saponified by refluxing in 20 mL of a 1 M KOH ethanol/water (8:2, v/v) solution for 1 h. The refluxed mixture was then transferred into a separatory funnel, and the reflux bottle was washed with 40 mL of Milli-Q water. The unsaponifiables in the combined solution were then extracted three times with 40 mL of diethyl ether. The ether phase was combined, washed three times with 40 mL of water, and dried with sodium sulfate overnight.

Isolation of Sterols and Preparation of TMS Derivatives. The unsaponifiables dissolved in petroleum ether (bp = 60−80 °C) were applied to a Silica Sep-Pak Cartridge (Waters Corp., Milford, MA) preconditioned with 5 mL of petroleum ether. After elution of hydrocarbons with 10 mL of petroleum ether and carotenoids with 10 mL of petroleum ether/ether (96:4, v/v), the tocopherols and tocotrienols were eluted with 10 mL of petroleum ether/ether (91:9, v/v) and sterols with 20 mL of petroleum ether/ether (1:1, v/v). The sterols were derivatized by incubation in a mixture of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (2:1, v/v), at 60 °C for 2 h.

Identification of Sterols. The sterol TMS derivatives were analyzed by gas chromatography−mass spectrometry (GC-MS). A VG Analytical 7070E double-focusing mass spectrometer (VG Analytical, Altrincham, U.K.) equipped with an Opus data system (Fisons Instruments, Manchester, U.K.) was connected to a Dani 3800HR 2Ch gas chromatograph (Dani S.p.A, Milan, Italy) without a restrictor capillary. A 30 m × 0.25 mm i.d., 0.25 μm film DB-1701 capillary column (J&W Scientific, Folsom, CA) was inserted directly into the ion source. The column temperature was held at 230 °C for 1 min, increased to 275 °C at 1 °C/min, and finally held at 275 °C for 30 min. Helium (1.5 mL/min) was used as a carrier gas. The injector temperature was 275 °C. The positive ion electron impact (EI) mass spectra were recorded at an ionization energy of 70 eV and a trap current of 100 μA with a source temperature of 180 °C. Perfluorokerosene (PFK) (Merck Art. 10145) was used as the reference compound. The magnetic sector scans were performed within the range of m/z 35−600 at a rate of 1 scan/s with an intermediate time of 0.5 s between the scans, an acceleration voltage of 6 kV, and a resolution of 1000.

Reference compounds, campesterol (campest-5-en-3β-ol), sitosterol (stigmast-5-en-3β-ol), stigmastanol (5α-stigmastan-3β-ol), α-amyrin (5α-urs-12-en-3β-ol), and β-amyrin (5α-olean-12-en-3β-ol) were purchased from Sigma Chemical Co. (St. Louis, MO). Sterols were identified by comparing the mass spectra and retention times with those of reference compounds, or the mass spectra published in the literature. For those compounds for which neither standard compounds nor reference spectra were available, the chemical structures were postulated according to the general patterns of mass spectrometric fragmentation of different sterols.

Quantatitive Analysis. The sterol TMS derivatives were analyzed with a Varian 3300 gas chromatograph equipped with a flame ionization detector (FID). The same column and GC parameters were used as in the GC-MS analysis. The FID temperature was 300 °C, and the split ratio of the injector was 1:40. The quantification of sterol compounds was carried out with a cholesterol internal standard and calculated by applying the detector reponse of sitosterol. The contents of sterols in seeds, pulp/peel, and whole berries were calculated according to the oil contents in these fractions and the contents of sterols in the oils. The repeatability of the analytical procedure was tested, and the relative standard deviation (SD) of five repeated analyses of a single sample was <5%.

Statistical Analysis. Statistical analyses were carried out with the statistical program SPSS 7.5. Comparison of the absolute contents and the proportions of different sterols in ssp. *sinensis* and *rhamnoides* were carried out with an independent sample t test and a Mann−Whitney U test.

RESULTS AND DISCUSSION

Sterols in Seeds and Pulp/Peel of Berries. Figure 1 presents GC-FID chromatograms of sterols in seeds and pulp/peel, respectively. Identification of the compounds in the chromatograms is summarized in Table 2. The same major sterols were found in the soft parts and in the seeds even though differences were found in minor compounds. In addition to sterols, a group of triterpenoids was found, especially in the pulp/peel (peaks 16 and 19−25), of which two compounds (peaks 24 and 25) had spectra identical to that reported and tentatively identified as friedelan-3-ol in grape seed oil by Kornfeldt (*22*). A detailed identification of these compounds was beyond the scope of the present study. Only sterols were included in the calculation of the percentages of different compounds.

Compounds **1**, **3**, **4**, and **8** were identified as TMS ether derivatives of campesterol, sitosterol, stigmastanol, and α-amyrin, respectively, by comparing the retention times and mass spectra with those of the TMS

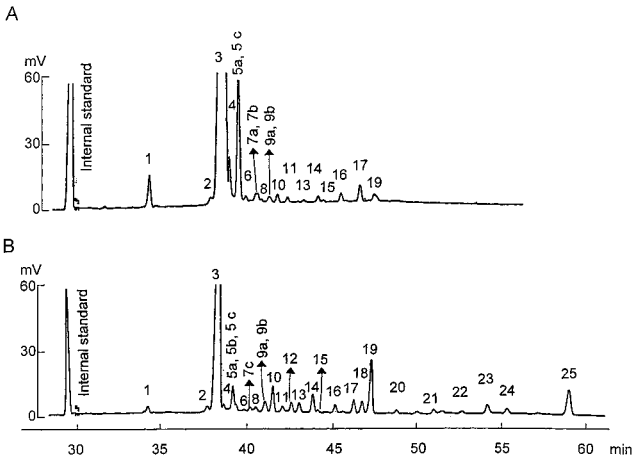

Figure 1. GC-FID chromatograms of sterols of seeds (A) and pulp/peel (B) of sea buckthorn berries (*H. rhamnoides* L. ssp. *rhamnoides*, r3 from Pyhämaa, Finland) analyzed as TMS deravatives.

ethers of reference compounds. These mass spectra also matched those published in the literature (*23*).

Peak 5 in seeds contained two compounds (**5a** and **5c**), and the major component **5a** was identifed as isofucosterol [24(*Z*)-stigmasta-5,24(24^1)-dien-3β-ol] according to its mass spectrum and retention time (*23*, *24*). The mass spectrum of **5c** ($\sim^1/_{10}$ of the abundancy of **5a**) matched well that of the TMS ether of obtusifoliol [4α,14α-dimethyl-5α-ergosta-8,24(24^1)-dien-3β-ol] (*22*), a key intermediate compound in the most common sterol biosynthesis pathways in plants (*25*). Thus, this compound was identified as obtusifoliol.

In the pulp/peel, a third compound, **5b**, was found at about the same level as **5a**. The strong molecular ion (*m/z* 486, base peak) and the weak ions at *m/z* 129 and 357 (M − 129) (Table 2) suggested that the compound was probably stigmast-8-en-3β-ol (*23*).

Peak 7 represents two compounds (**7a** and **7b**) in the seeds and is different from the corresponding one (**7c**) in the pulp/peel. Compound **7a** was possibly a 14α-methyl-Δ^8-sterol or a 14α-methyl-$\Delta^{9(11)}$-sterol (*23*). Compound **7b** gave a spectrum similar to that of **5a**, characteristic of $\Delta^{5,24(24^1)}$-sterols and 24-ethyl-$\Delta^{5,24(25)}$-sterols. According to Goad and Akihisa (*23*), the latter group tends to have longer retention times, and thus compound **7b** was possibly stigmasta-5,24(25)-dien-3β-ol. Compound **7c** was possibly a stigmasta-8,24-dien-3β-ol (*23*, *26*). However, the final identification of this compound has to be carried out with reference compounds because of the lack of diagnostic ions in the spectra of Δ^8-sterols (*23*).

The mass spectrum of compound **9a** matched well the spectrum of TMS ether of schottenol (5α-stigmast-7-en-3β-ol) published in the literature (*23*) and was identified as stigmast-7-en-3β-ol.

Compound **9b** had a molecular ion at *m/z* 498 in its mass spectrum. The ions at *m/z* 483 and the strong ions at *m/z* 408 (M − 90), *m/z* 393 (M − 90 − 15), *m/z* 286 (M − 90 − A-ring − C-6), *m/z* 339 (due to the loss of TMSiOH, C-2, C-3, C-4, C-28, and C-29), and *m/z* 365 (M − 90 − 43) suggested that the compound had a 9β,-19-cyclopropane structure in the ring system, a Δ^{24}-unsubstituted side chain, and a C-14 methyl group. The mass spectrum matched well the published spectrum of the TMS ether of cycloartenol (9,19-cyclo-5α,9β-lanost-24-en-3β-ol) (*23*, *27*). Therefore, compound **9b** was identified as cycloartenol. Cycloartenol is commonly known as the precursor of other sterols in plants. A third compound was also found in the same peak in seeds but was not identified because of its strong overlapping with the other compounds.

Peak 10 had a mass spectrum similar to that of cycloartenol. The ions at *m/z* 353 (339 + 14) and 300 (286 + 14) were characteristic of 9β,19-cyclopropane sterols with a 24-methylene side chain (*23*). The compound in peak 10 was suggested to be 4α,14α-dimethyl-9β,19-cycloergost-24(24^1)-en-3β-ol. A minor component with a molecular weight of 512 was also found in peak 10 and was not identified because of its small amount and strong overlapping with the major component.

The compound in peak 11 was identified as stigmasta-7,24(24^1)-dien-3β-ol on the basis of the close match of its mass spectrum with that of avenasterol [(24Z)-5α-stigmasta-7,24(24^1)-dien-3β-ol] TMS ether published by Knights (*26*).

Compound **12** was found only in the pulp/peel of the berries. Its mass spectrum was similar to those of compound **10** and cycloartenol, suggesting a cyclopropane structure in the fused ring system. However, the absence of diagnostic ions made further indentification of the compound impossible.

The spectrum of compound **13** fitted with those of 14α-methyl-Δ^8-sterols and 14α-methyl-$\Delta^{9(11)}$-sterols. The ion at *m/z* 309 (M − 105 − 98) suggested the existence of a 24-ethylidene or 24-ethyl-Δ^{24} side chain. Ge (*17*) reported 4,14,24^1-trimethylergosta-8,24-dien-3β-ol in sea buckthorn pulp oil. Compound **13** was possibly 4,14,-24^1-trimethylergosta-8,24-dien-3β-ol, but further investigation is needed to confirm this tentative identification.

Compound **14** was identified as 24-methylenecycloartanol according to its mass spectrum matching well that of the TMS ether of 24-methylenecycloartanol [24-methyl-5α-cycloart-24(24^1)-en-3β-ol] published by Soulier et al. (*27*).

Compound **17** in pulp/peel was identified as citrostadienol [(24*Z*)-4α-methyl-5α-stigmasta-7,24(24^1)-dien-3β-ol] according to its mass spectrum, which was identical to that of the TMS ether of citrostadienol published by Farines et al. (*24*).

The structure of compound **18** could not be defined, although its spectrum suggested a 9,19-cyclopropane structure in the fused ring system.

The structures of minor components **2**, **6**, and **15** cannot be defined according to their spectra alone.

Salenko et al. (*13*) analyzed the unsaponifiable fraction of a pentane extract of the fruit pulp of sea buckthorn, and sitosterol, 24-methylenecycloartenol, citrostadienol, 24-ethylcholest-7-en-3β-ol, α- and β-amyrins (pentacyclic triterpenes), uvaol, and erythrodiol were identified with MS, GC-MS, and NMR. Sitosterol, 24-methylenecycloartenol, citrostadienol, and α-amyrin were also found in the present study. The "24-ethylcholest-7-en-3β-ol" identified by Salenko et al. (*13*) was probably the same compound as the stigmast-7-en-3β-ol in our study. We did not find β-amyrin in the analyzed samples.

Ge (*17*) reported 19 sterol compounds in pulp oil of sea buckthorn (*H. rhamnoides* ssp. *sinensis*) with GC-MS, among which sitosterol, α-amyrin, and stigmast-7-en-3β-ol were also found in our study. Other compounds reported were β-amyrin, ergosta-5,8,22-trien-3β-ol, ergosta-5-en-3β-ol, ergosta-7-en-3β-ol, 4,14-dimethyl-

Table 2. Sterols and Triterpenes in Seeds and Pulp/Peel of Sea Buckthorn Berries (Mass Spectra Shown Are of Sterol TMS Ethers)

compd	RRT[a]	major ions in mass spectra (*m/z*, intensity relative to base peak	identification	occurrence	ID method[b]	refs
1	1.16	472 (62), 457 (13), 382 (85), 367 (33), 343 (96), 261 (11), 255 (16), 227 (4), 213 (11), 129 (100)	campest-5-en-3β-ol (campesterol)	seeds, pulp/peel	S, R	*14*, *17*−*19*
2	1.28	484 (100), 469 (34), 386 (30), 379 (85), 355 (11), 296 (13), 253 (38), 213 (15), 211 (22), 129 (48)	stigmastadienol[c]	seeds, pulp/peel		
3	1.30	486 (65), 471 (13), 396 (91), 381 (30), 357 (93), 255 (14), 228 (2), 213 (9), 129 (100)	stigmast-5-en-3β-ol (sitosterol)	seeds, pulp/peel	S, R	*13*−*15*, *17*−*19*
4	1.31	488 (93), 473 (53), 431 (9), 398 (24), 383 (24), 357 (11), 305 (22), 290 (8), 230 (19), 215 (60), 75 (100)	5α-stigmastan-3β-ol (stigmastanol)	seeds, pulp/peel	S, R	
5a	1.33	484 (15), 469 (7), 394 (5), 386 (100), 371 (12), 355 (9), 296 (57), 281 (22), 257 (22), 255 (7), 227 (6), 213 (8), 211 (8), 129 (50)	24(*Z*)-stigmasta-5,24(24^1)-dien-3β-ol (isofucosterol)	seeds, pulp/peel	D, R	*14*, *18*, *19*
5b		486 (100), 471 (19), 396 (6), 393 (18), 381 (12), 345 (6), 255 (17), 229 (16), 213 (20), 201 (11)	stigmast-8-en-3β-ol[c]	pulp/peel	D, R	
5c		498 (65), 483 (100), 455 (2), 393 (32), 317 (5), 295 (11)	4α,14α-dimethyl-5α-ergosta-8,24(24^1)-dien-3β-ol (obtusifoliol)	seeds, pulp/peel	D, R	*15*−*18*
6	1.35	500 (5), 486 (7), 471 (6), 388 (100), 373 (13), 305 (10), 215 (16), 75 (37)	not identified	seeds, pulp/peel		
7a	1.36	500 (52), 485 (51), 483 (32), 469 (26), 410 (50), 395 (100), 371 (65), 355 (8), 343 (5), 303 (5), 269 (6), 229 (17), 213 (23), 187 (33)	not identified	seeds		
7b		484 (37), 469 (14), 394 (9), 386 (100), 371 (4), 355 (24), 296 (58), 281 (22), 257 (23), 255 (9), 227 (8), 213 (12), 211 (9), 129 (68)	stigmasta-5,24(25)-dien-3β-ol[c]	seeds	D	
7c		484 (100), 469 (32), 394 (6), 386 (15), 379 (12), 343 (9), 281 (5), 255 (7), 229 (16), 213 (20)	stigmasta-(8,24)-dien-3β-ol[c]	pulp/peel	D	
8	1.38	498 (13), 484 (8), 408 (5), 393 (4), 388 (6), 279 (6), 269 (4), 218 (100), 207 (6), 203 (10)	5α-urs-12-en-3β-ol (α-amyrin)	seeds, pulp/peel	S, R	*13*, *15*−*18*
9a	1.39	486 (100), 471 (15), 396 (4), 381 (9), 345 (5), 303 (4), 255 (45), 229 (11), 213 (12)	stigmast-7-en-3β-ol	seeqds, pulp/peel	D, R	*13*−*15*, *17*−*19*
9b		498 (2), 483 (11), 408 (100), 393 (45), 365 (37), 339 (38), 297 (5), 286 (20), 271 (9), 69 (63)	9,19-cylco-5α,9β-lanost-24-en-3β-ol (cycloartenol)	seeds, pulp/peel	D, R	*18*
10	1.41	498 (2), 483 (3), 408 (100), 393 (42), 365 (2), 353 (6), 300 (4), 283 (7), 269 (3), 217 (5), 189 (10)	4α,14α-dimethyl-9β,19-cyclo-ergost-24(24^1)-en-3β-ol[c]	seeds, pulp/peel	D	
11	1.43	484 (21), 469 (10), 394 (3), 386 (100), 371 (8), 343 (88), 296 (6), 281 (6), 255 (13), 253 (8), 229 (5), 211 (3)	stigmasta-7,24(24^1)-dien-3β-ol	seeds, pulp/peel	D, R	*14*, *19*
12	1.45	498 (4), 483 (6), 408 (100), 393 (44), 311 (12), 297 (3), 269 (12), 216 (15), 201 (6)	not identified	pulp/peel		
13	1.46	512 (81), 497 (100), 485 (7), 458 (4), 407 (59), 368 (13), 353 (3), 329 (5), 309 (17), 278 (7), 255 (3), 215 (12), 213 (11), 69 (57)	4α,14α,24^1-trimethylergosta-8,24(24^1)-dien-3β-ol[c]	seeds, pulp/peel	D	*17*
14	1.49	512 (4), 497 (8), 422 (100), 407 (40), 379 (33), 353 (15), 339 (3), 323 (2), 300 (13), 297 (6), 255 (3), 241 (3)	24-methyl-5α-cycloart-24(24^1)-en-3β-ol (24-methylenecycloartanol)	seeds, pulp/peel	D, R	*13*, *18*
15	1.50	498 (100), 483 (18), 422 (15), 408 (10), 396 (53), 391 (15), 383 (11), 356 (7), 342 (6), 269 (6), 253 (11), 243 (8), 241 (9), 227 (15)	not identified	seeds, pulp/peel		
16	1.53	571 (2), 496 (100), 481 (3), 391 (2), 216 (83), 203 (34), 189 (15), 133 (7), 73 (41)	unidentified triterpene, isomer of **19**	seeds, pulp/peel		
17	1.57	498 (10), 483 (9), 400 (100), 393 (5), 385 (5), 357 (71), 310 (7), 295 (6), 267 (9), 241 (8), 227 (11)	24(*Z*)-4α-methyl-5α-stigmasta-7,24(24^1)-dien-3β-ol (citrostadienol)	seeds, pulp/peel	D, R	*13*
18	1.58	512 (2), 422 (100), 407 (45), 379 (1), 367 (7), 325 (11), 312 (3), 309 (2), 283 (7), 69 (29)	not identified	pulp/peel		
19	1.60	571 (3), 496 (100), 481 (2), 393 (2), 391 (2), 216 (37), 203 (36), 188 (13), 133 (11), 95 (12), 73 (37)	unidentified triterpene, isomer of **16**	seeds, pulp/peel		
20	1.65	586 (7), 571 (4), 496 (85), 483 (57), 393 (29), 374 (10), 279 (13), 203 (56), 189 (56), 129 (28)	unidentified	pulp/peel		
21	1.73	586 (12), 482 (31), 320 (50), 307 (5), 279 (8), 219 (5), 203 (100), 189 (30), 133 (8), 73 (64)	unidentified triterpene, isomer of **23**	pulp/peel		
22	1.78	587 (5), 496 (2), 481 (2), 279 (6), 216 (100), 201 (11), 189 (11), 145 (10), 120 (46), 73 (25)	unidentified triterpene	pulp/peel		
23	1.83	586 (11), 512 (2), 482 (13), 320 (100), 307 (10), 279 (12), 203 (91), 189 (29), 133 (32), 73 (74)	unidentified triterpene, isomer of **21**	pulp/peel		
24	1.88	512 (5), 497 (2), 407 (1), 422 (2), 279 (15), 232 (69), 203 (100), 190 (57), 175 (13), 131 (18)	friedelan-3-ol[c]	pulp/peel	R	
25	2.00	512 (5), 497 (2), 407 (1), 422 (2), 279 (17), 232 (22), 203 (100), 190 (47), 175 (9), 133 (19)	friedelan-3-ol[c]	pulp/peel	R	

[a] Retention time relative to cholesterol. [b] S, identified with standard compound; R, identified with published reference spectrum; D, deduced according to the mass spectrum in the present study. [c] Tentative identification.

ergosta-8,24(28)-dien-3β-ol [4,14-dimethylergosta-8,24-(24^1)-dien-3β-ol], stigmasta-5-en-3-one, lanosta-8,24-dien-3β-ol, 9,19-cyclolanosta-25-en-3β-ol series (three separated compounds), stigmasta-7,16-dien-3β-ol, 24-methylenelanosta-8-en-3β-ol, 4,14,28-trimethylergosta-8,24(28)-dien-3β-ol [4,14,28-trimethylergosta-8,24(24^1)-dien-3β-ol], 4-methylstigmasta-7,16-dien-3β-ol, 14-methyl-α-sitosterol, and 14,15-dimethyl-α-sitosterol. According to the structure presented in the publication, the last two compounds were 4,14-dimethylstigmasta-7,24(24^1)-

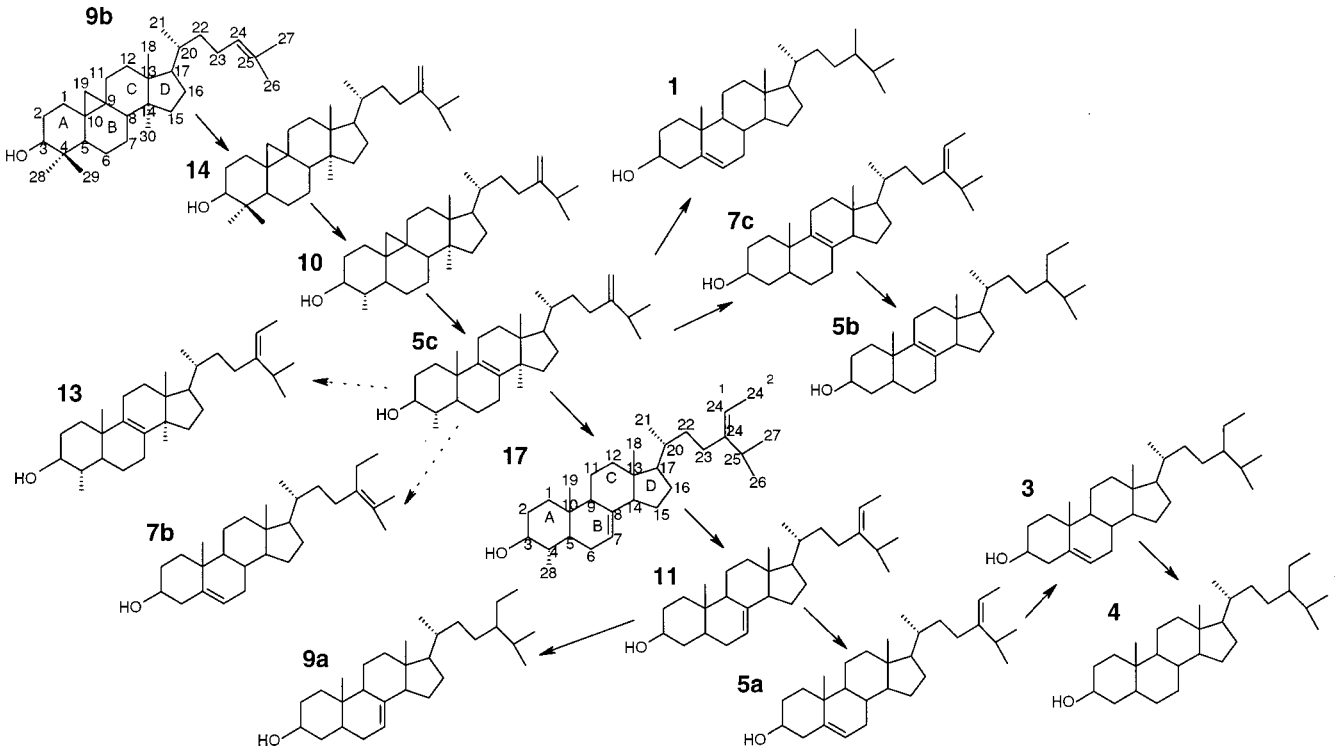

Figure 2. Major sterols found in sea buckthorn seeds and berries and possible precursor−product relationships between these compounds. Arrows with solid tails represent the dominating pathways proposed in plants; arrows with dotted tails represent less common pathways.

dien-ol and 4,14,15-trimethylstigmasta-7,24(24[1])-dien-ol, respectively (*17*).

4,14-Dimethyl-ergosta-8,24(24[1])-dien-3β-ol and 4,14,-28-trimethylergosta-8,24(24[1])-dien-3β-ol in Ge's identification were possibly the same as compounds **5c** (obtusifoliol) and **13** in the present study, respectively. The mass spectrum of "4-methylstigmasta-7,16-dien-3β-ol" in the publication (*17*) resembled that of citrostadienol published in the literature (*28*), and it is possible that this compound was actually citrostadienol. The "ergost-5-en-3β-ol" was probably the same compound as campesterol [24(*R*)-ergost-5-en-3β-ol or campest-5- en-3β-ol] identified in the present study.

Wang et al. (*15*) reported 22 sterols and triterpenes in pulp oil and 10 in seed oil of sea buckthorn ssp. *sinensis*. Only α- and β-amyrins, 4,14-dimethyl-ergosta-8,-24(24[1])-dien-3β-ol, and 9,19-cyclolanosta-25-en-3β-ol were claimed to be the common components found in both seed and pulp oils. Sitosterol was reported to be absent in the seed oil. In contrast to this, we found that the sterol compositions in seed and pulp/peel of sea buckthorn berries were rather similar, except for a few minor components, and sitosterol was always the most abundant component (60−80%) in both seeds and pulp/peel.

Schiller (*14*) reported sitosterol (86% of fruit pulp sterols, 73% of seeds sterols), campesterol (2% of fruit pulp sterols and 4% of seed sterols), stigmasta-5,24-dienol (6% in fruit pulp sterols and 17% in seed sterols), stigmast-7-en-ol (2% in fruit pulp sterols and 1% in seeds sterols), stigmasta-7,24-dien-ol (1% in fruit pulp and seed sterols), a small amount of cholesterol (1.5−2.0%) in seeds and fruit flesh, and a trace amount of stigmasterol (0.1% of total sterols) in berry pulp. The same compounds were found by Bat (*19*) in oils from seeds, fruit flesh, and press cake of sea buckthorn berries. Compared with those of Wang et al. (*15*) and Ge (*17*), the identifications of Salenko et al. (*13*), Schiller (*14*), and Bat (*19*) were more consistent with that in the present study, although we did not find detectable amount of cholesterol or stigmasterol. The discrepancy among the identifications in the investigations suggested cautions should be excercized when sterol compositions are adopted as a criterion for assessing adulteration of sea buckthorn oils.

In addition to the compounds reported earlier in sea buckthorn, five compounds were identified (or postulated) in the present study, of which the occurrence in sea buckthorn seeds and berries is reported for the first time (Table 2).

A dominating biosynthetic pathway of sterols has been proposed to operate in most vascular plants based on the sterols identified and the enzymological investigations of sterol biosynthesis in a number of plant species (*25*, *29*). Deviations from the proposed biosynthetic pathway have also been suggested in some species (*25*, *29*). The differences found in the biosynthesis of sterols in different plant species are possibly related to the phylogenetical regulation of the expression of genes coding for some enzymes involved in the process (*29*). The biosynthesis of sterols in sea buckthorn has not been investigated or discussed before. On the basis of the existing knowledge of biosynthesis of plant sterols, the major sterols identified in the present study are presented in a scheme as Figure 2, suggesting the possible precursor−product relationships between these compounds. The scheme is in agreement with the dominating pathway proposed by Benveniste (*25*) and Nes and Venkatramesh (*29*). Further investigations are needed to demonstrate the biosynthesis of sterols in sea buckthorn.

Sterol Content in Seeds. Table 3 presents the sterol contents in seeds of different origins of the two subspe-

Table 3. Sterol Content (Milligrams per Kilogram) in Seeds of Two Subspecies of Sea Buckthorn

	sample								subspecies				
									sinensis		*rhamnoides*		
sterol	s1	s2	s3	s4	r1	r2	r3	r4	mean	SD	mean	SD	*p* value
1	31	32	39	31	35	37	51	35	33	4	40	8	0.20
3	992	805	1041	896	763	822	1164	976	934	105	931	179	0.98
4	52	53	30	49	59	56	70	16	46	11	50	24	0.75
5a + **5c**	243	292	166	276	189	160	282	172	244	56	201	55	0.31
6	39		49	12	5	3	8	3	33	19	5	2	**0.03**
7a + **7b**	21	27	17	23	17	27	14	10	22	4	17	7	0.28
9a + **9b**	24	39	11	25	10	25	34	6	25	11	19	13	0.52
10	12	18	9	10	13	15	29	9	12	4	17	9	0.41
11	26	35	18	23	12	14	14	7	26	7	12	3	**0.01**
13	9	8	5	7	10	7	9	3	7	2	7	3	1.00
14	20	39	10	16	13	43	48	11	21	13	29	19	0.54
15	6	1	3	4	6	2	2	6	4	2	4	2	0.76
17	44	64	24	37	30	40	58	26	42	17	39	14	0.74
total	1519	1413	1422	1409	1162	1251	1783	1280	1441	52	1369	281	0.63
α-amyrin and unidentified triterpenes													
8	7	12	6	8	6	11	7	2	8	3	7	4	0.47
16	13	8	9	10	9	12	11	17	10	2	12	3	0.31
19	36	13	7	15	7	9	9	23	18	13	12	7	0.46
total	56	33	22	33	22	32	27	42	36	14	31	9	0.55

Table 4. Sterol Content (Milligrams per Kilogram) in Fresh Pulp/Peel of Berries of Two Subspecies of Sea Buckthorn

	sample								subspecies				
									sinensis		*rhamnoides*		
sterol	s1	s2	s3	s4	r1	r2	r3	r4	mean	SD	mean	SD	*p* value
1	6	3	5	5	4	4	3	4	4.8	1.3	3.8	0.5	0.19
2	7	4	6	5	3	3	4	3	5.5	1.3	3.3	0.5	**0.02**
3	328	164	196	186	183	225	211	173	218.5	74.2	198.0	24.1	0.62
4	4	1	1	3	1	1	2	1	2.3	1.5	1.3	0.5	0.25
5a + **5b** + **5c**	10	14	17	20	8	28	22	17	15.3	4.3	18.8	8.5	0.49
6					5		9				7.0	2.8	
7a + **7b** + **7c**	2	3	6	4		7		3	3.8	1.7	5.0	2.8	0.52
9a + **9b**	9	7	8	10	3	6	6	6	8.5	1.3	5.3	1.5	**0.02**
10	3	10	18	10	4	5	6	11	10.3	6.1	6.5	3.1	0.32
11	7.3	5	8	10	4	7	7	4	7.6	2.1	5.5	1.7	0.17
12	4	4	6	6	2	3	9	5	5.0	1.2	4.8	3.1	0.88
13	2	4	8	5	9	6	6	5	4.8	2.5	6.5	1.7	0.29
14	3	7	19	8	5	4	9	8	9.3	6.8	6.5	2.4	0.48
15					4	4		2			3.3	1.2	
17	6	6	12	6	5	7	5	6	7.5	2.0	5.8	1.0	0.31
18	6	5	9	6	3	3	6	6	6.5	1.7	4.5	1.7	0.15
total	397.3	237	319	284	243	313	305	254	309.3	67.6	278.8	35.4	0.45
α-amyrin and unidentified triterpenes													
8	0	3	3	2	4	5	7	3	2.0	1.4	4.8	1.7	**0.05**
16	19	6	6	8	6	4	11	5	9.8	6.2	6.5	3.1	0.39
19	88.1	27	19	37	34	18	58	22	42.8	31.1	33.0	18.0	0.61
20	4	1		3	2	1	4	1	2.7	1.5	2.0	1.4	0.58
21	55	14	8	30	8	5	5	2	24.0	21.0	5.0	2.4	0.09
22	9	3			3	3	3	2	6.0	4.2	2.8	0.5	0.16
23	121	15	11	64	8	6	8	6	52.8	51.5	7.0	1.2	0.13
24	3	7	6	2	7	5	4	3	4.5	2.4	4.8	1.7	0.87
25	18	12	31	14	30	24	13	14	18.8	8.5	20.3	8.2	0.81
total	317.1	88	84	160	102	71	113	58	162.3	109.0	86.0	25.8	0.22

cies. In the seed samples analyzed, the total sterol content varied from 1200 to 1800 mg/kg, of which 57−76% was sitosterol and 13−21% isofucosterol. Stigmastanol, citrostadienol, and campesterol constituted 1−5, 2−5, and 2−3% of total seed sterols, repectively. The proportion of the other sterols was typically 1−2% each. No significant difference was found in the level of most of the compounds or the total sterols between the two subspecies. The contents of compound **6** (33 ± 19 vs 5 ± 2 mg/kg, $p < 0.05$) and stigmasta-7,24(24[1])-dien-3β-ol (compound **11**) (26 ± 7 vs 12 ± 3 mg/kg, $p < 0.05$) were higher in seeds of ssp. *sinensis* than in those of ssp. *rhamnoides*. In the extracted seed oil, the total sterol content was 12.4−23.0 g/kg in the samples analyzed.

Comparison of the sterol composition in seeds of the two subspecies also showed that the proportions of most of the sterols were similar in the two subspecies even though the berries have been collected in different years from growth sites with different geographic, soil, and climate conditions. Only compounds **6** (2.3 vs 0.4%) and **11** [stigmasta-7,24(24[1])-dien-3β-ol] (1.8 vs 0.9%) represented a higher proportion of total sterols in ssp. *sinensis* than in ssp. *rhamnoides* ($p < 0.05$).

From the end of August to the end of November 1998, an increase was found in the content of campesterol (compound **1**), whereas the levels of stigmastanol (compound **4**), compound **6**, and stigmasta-7,24(24[1])-dien-3β-ol (compound **11**) decreased (Figure 3A1) in seeds of ssp. *sinensis*. The sum of isofucosterol and obtusifoliol (compounds **5a** + **5c**) reached a maximum at mid-October (Figure 3A2). The total sterol content and the level of sitosterol (compound **3**) remained rather constant, even though the values at mid-October were

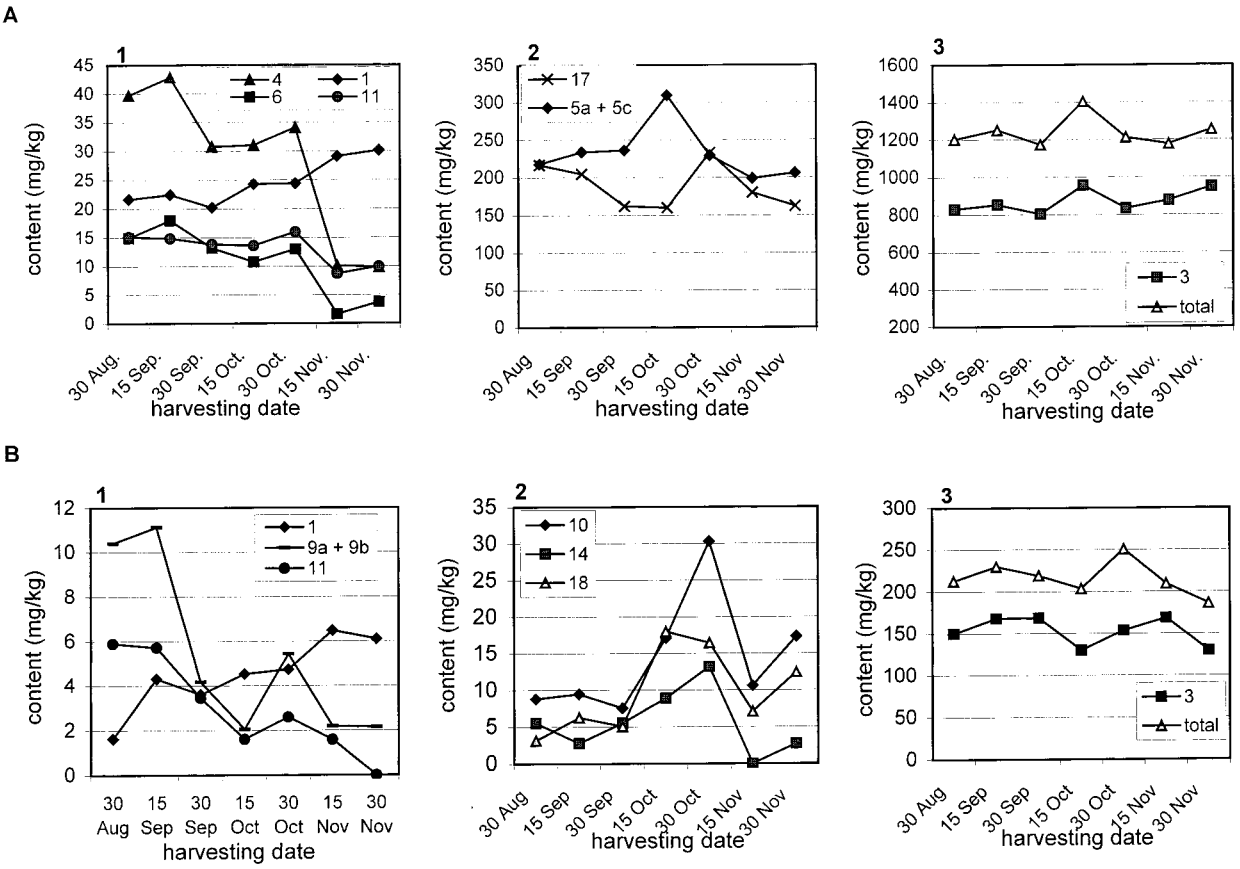

Figure 3. Changes in sterol contents in seeds (A) and pulp/peel (B) of sea buckthorn (*H. rhanmoides* L. ssp. *sinensis*) growing in Xixian County, People's Republic of China, from the end of August to the end of November 1998.

slightly higher than those at other dates (Figure 3A3). During the period of investigation, the proportions of sitosterol (from 69 to 76%) and campesterol (from 1.8 to 2.4%) of total seed sterols increased, whereas those of stigmastanol (from 3.3 to 0.8%) and compound *6* (from 1.2 to 0.3%) decreased. The proportions of other compounds remained fairly constant.

The results show that, like many other components, the levels of most sterols in the seeds of ripe berries remained rather constant during the harvesting period. The clear increase in campesterol and decrease in stigmastanol suggested that a complete balance was not yet reached in the major biosynthetic pathways at the beginning of the harvesting period.

The total content of α-amyrin and the unidentified triterpenes in the seeds varied from 22 to 56 mg/kg without any significant difference between the two subspecies (Table 3).

Sterol Content in Pulp/Peel of Berries. Table 4 presents the contents of sterols in pulp/peel of the berries. The total sterol content in the fresh soft parts varied within the range of 240−400 mg/kg. The total sterol content in the extracted pulp/peel oil was 10.3−28.7 g/kg in the analyzed samples. Sitosterol constituted 61−83% of the total sterols in the soft parts of the berries. Isofucosterol, stigmast-8-en-3β-ol, and obtusifoliol together (compounds **5a** + **5b** + **5c**) comprised 3−9%, showing a significantly lower proportion of isofucosterol than in the seeds. This was consistent with the results (17% of seed sterols and 6% of fruit pulp sterols were stigmasta-5,24-dien-ol) of Schiller (*29*). The proportions of the rest were typically 1−3% each of the total sterols.

When the two subspecies were compared, no significant difference was found in the absolute content or the proportion of sitosterol and most of the other sterols in the soft parts. The absolute levels of compound **2** (a stigmastadienol) and the sum of stigmasta-7-en-3β-ol and cycloartenol (compounds **9a** + **9b**) were higher in ssp. *sinensis* ($p < 0.05$). In addition, the unidentified compound **15** was not detected in this subspecies (Table 4).

The total content of the unidentified triterpenes varied from 58 to 317 mg/kg in the fresh pulp/peel of the berries. The average level was higher in ssp. *sinensis* (162 vs 86 mg/kg). However, the difference was not statistically significant because of the high deviation (Table 4).

From the end of August to the end of November 1998, the level of campesterol (compound **1**) increased, whereas the contents of stigmasta-7,24(24^1)-dien-3β-ol (compound **11**) and the sum of stigmast-7-en-3β-ol and cycloartenol (compounds **9a** + **9b**) decreased significantly (Figure 3B1). A significant decrease was also found in the sum of isofucosterol, stigmast-8-en-ol, and obtusifoliol (compounds **5a** + **5b** + **5c**). The levels of 4α,14α-dimethyl-9β,19-cycloergost-24(24^1)-en-3β-ol (compound **10**), 24-methylenecycloartanol (compound **14**), and compound **18** reached a maximum at the end of October (Figure 3B2). A slight decrease was found both in the total sterol content (from 213 to 186 mg/kg) and in the content of sitosterol (from 150 to 130 mg/kg) of pulp/peel during this period (Figure 3B3).

Sterol Content in Whole Berries. Table 5 presents the sterol contents in fresh whole berries of different origins. The total sterol contents of the samples varied

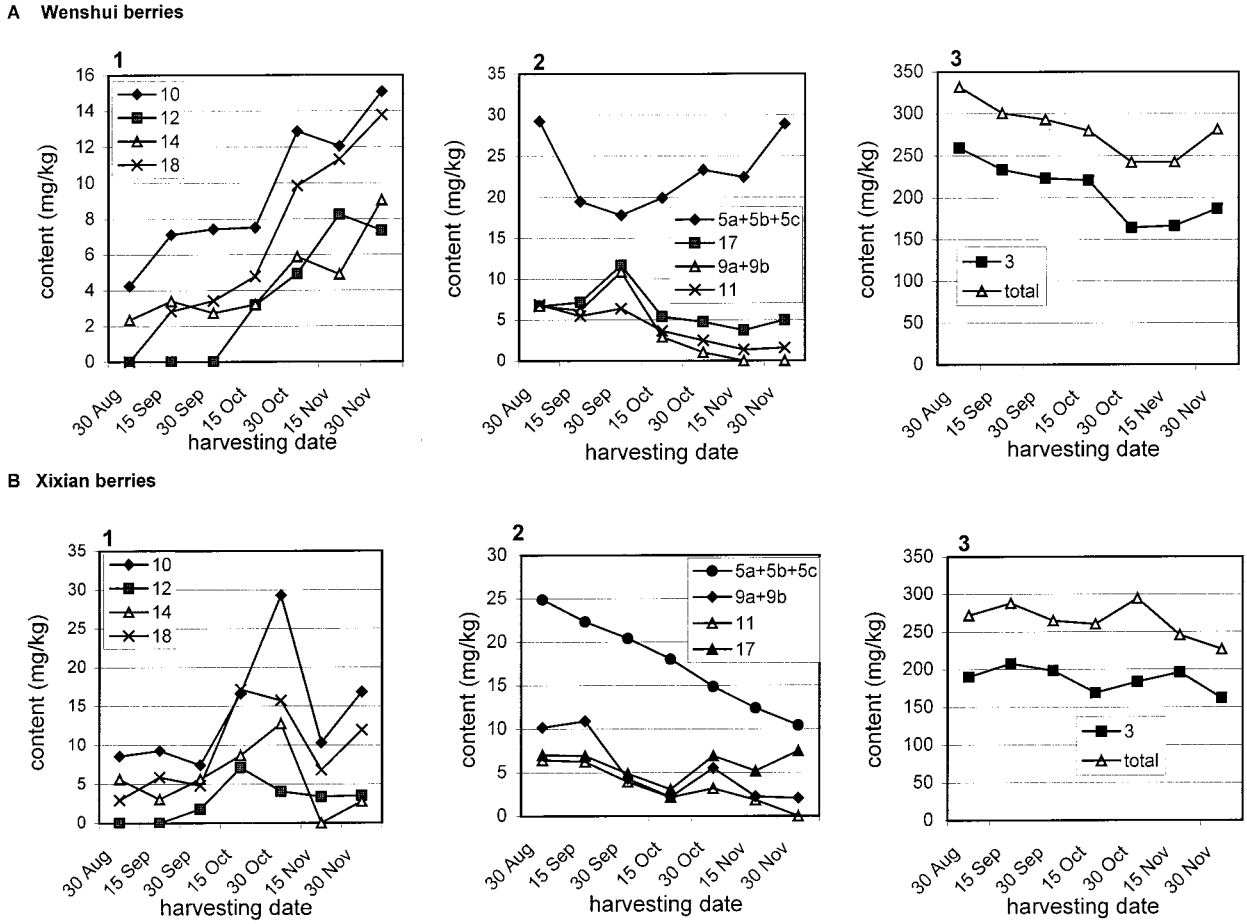

Figure 4. Changes in sterol contents in fresh whole berries of sea buckthorn growing in Wenshui (A) and Xixian (B) counties, People's Republic of China, from the end of August to the end of November 1998.

Table 5. Sterol Content (Milligrams per Kilogram) in Fresh Berries

	sample								subspecies *sinensis*		subspecies *rhamnoides*		
sterol	s1	s2	s3	s4	r1	r2	r3	r4	mean	SD	mean	SD	*p* value
1	10	5	9	7	7	7	6	6	8	2	7	1	0.32
3	317	215	309	257	243	247	299	233	275	48	256	30	0.52
4	7	2	2	4	1	1	2	2	4	2	2	1	0.11
5a + 5b + 5c	66	43	37	42	28	31	42	27	47	13	32	7	**0.09**
6	1	1		1		9			1	0	9		
7c		2	7	6	6		6	5	5	3	6	1	0.69
9a + 9b	19	11	8	10	10	8	10	7	12	5	9	2	0.25
10	13	15	16	16	9	10	11	15	15	1	11	3	**0.05**
11	21	9	9	9	4	5	7	5	12	6	5	1	**0.07**
12	10	5	6	5	2	2	8	5	7	2	4	3	0.27
13	9	8	6	7	8	8	11	6	8	1	8	2	0.56
14	10	14	12	15	13	11	16	10	13	2	13	3	0.89
15	7		1		4	5	2	3	4	4	4	1	0.82
17	14	16	15	13	16	11	19	13	15	1	15	4	0.90
18	11	9	7	7	3	3	12	7	9	2	6	4	0.37
total	515	355	444	399	354	358	451	344	432	94	385	60	0.27
α-amyrin and triterpenes													
8	10	2	3	3	7	4	7	3	5	4	5	2	0.74
16	32	11	6	8	4	4	8	5	14	12	5	2	0.19
19	119	37	14	24	17	13	35	22	49	48	22	10	0.32
20	5	1	1	2	2	2	2	2	2	2	2	0	0.80
21	72	26	14	13	9	8	6	3	31.3	27.8	6.5	2.6	0.13
22	18	4	2	3	1	2	4	2	7	8	2	1	0.28
23	169	30	19	16	11	7	9	5	58.5	73.9	8	2.6	0.22
24	3	14	4	5	5	5	9	5	7	5	6	2	0.86
25	18	82	7	8	22	16	25	18	28.8	35.8	20.25	4.0	0.65
total	436	205	67	79	71	57	98	62	196.8	171.3	72	18.3	0.20

within the ranges of 340−520 mg/kg and 13.0−33.2 g/kg in fresh whole berries and the extracted berry oil, respectively. Comparison of the two subspecies showed that the total sterol content in ssp. *sinensis* berries was typically higher than in ssp. *rhamnoides* (432 ± 94 vs 385 ± 60 mg/kg), but the difference was not statistically

significant ($p = 0.27$). Difference between the two subspecies was found in the combined level of isofucosterol, stigmast-8-en-3β-ol, and obtusifoliol (compounds **5a** + **5b** + **5c**, $p = 0.09$) and the contents of 4,14-dimethyl-9,19-cyclo-ergost-24(24^1)-en-3β-ol (compound **10**, $p < 0.05$) and stigmasta-7,24(24^1)-dien-3β-ol (compound **11**, $p = 0.07$), the levels of these compounds being higher in ssp. *sinensis* berries.

The total contents of α-amyrin and the unidentified triterpenes in the whole berries showed considerable variation (from 57 to 436 mg/kg). The difference between the two subspecies (197 ± 171 vs 72 ± 18 mg/kg) was not statistically significant ($p = 0.20$).

From the end of August to the end of November 1998, a clear increase was found in the contents of 4,14-dimethyl-9,19-cycloergost-24(24^1)-en-3β-ol (compound **10**) and 24-methylenecycloartanol (compound **14**), as well as two unidentified compounds (**12** and **18**) in Wenshui berries (Figure 4A1). A generally decreasing trend was found in the levels of stigmast-7-en-3β-ol + cycloartenol (**9a** + **9b**), stigmast-7,24(24^1)-dien-3β-ol (compound **11**), and citrostadienol (compound **17**), even though citrostadienol and the sum of stigmast-7-en-3β-ol and cycloartenol had their maxima at the end of September (Figure 4A2). During the same period, the total sterol content and the content of sitosterol decreased from 332 to 281 mg/kg and from 259 to 187 mg/kg, respectively (Figure 4A3). No clear trend was found in the other compounds.

During the same period, in Xixian berries (Figure 4B), the levels of 4,14-dimethyl-9,19-cycloergost-24 (24^1)-en-3β-ol (compound **10**), 24-methylenecycloartanol (compound **14**), and unidentified compunds **12** and **18** reached maxima around the middle to the end of October (Figure 4B1). Clear decreases (60−100%) were found in the levels of stigmastanol (compound **4**); stigmasta-5,24-dien-β-ol, stigmast-8-en-3β-ol, and obtusifoliol (**5a** + **5b** + **5c**); stigmast-7-en-3β-ol and cycloartenol (**9a** + **9b**); and stigmasta-7,24(24^1)-dien-3β-ol (compound **11**) (Figure 4B2). The total sterol content and the content of sitosterol decreased from 271 to 226 mg/kg and from 190 to 161 mg/kg, respectively. The changing trends of the proportions of the sterols paralleled those of the absolute levels of these compounds for both Wenshui and Xixian berries. During the harvesting period examined, a common increase was found in the levels of the compounds in the earlier part of the major biosynthetic pathway, accompained by a decrease in the compounds in the later part, including sitosterol (Figure 4).

Among the different plant sterols, sitosterol and stigmastanol (sitostanol) have been most intensively investigated with respect to their physiological effects in man. Many beneficial effects have been shown for the two sterols (*30*−*36*). The results of the present study showed that within the investigated period, the end of August was the best time to harvest the berries to obtain a high content of both compounds in both seeds and whole berries. The information obtained in the present investigation is very useful for characterizing sea buckthorn oils, detecting adulterations, and further chemical and nutritional investigations of sterols in sea buckthorn. The results are also important for industrial utilization of the berries.

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