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Phytosterol content of sea buckthorn (*Hippophae rhamnoides* L.) seed oil: Extraction and identification

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Abstract

Phytosterols in sea buckthorn (*Hippophae rhamnoides* L.) seed oil extracted by cold pressing, hexane, and supercritical carbon dioxide were identified by GC–MS and FID. Compounds identified were campesterol, clerosterol, lanosterol, sitosterol, β-amyrin, sitostanol, Δ^5-avenasterol, $\Delta^{24(28)}$-stigmasta-en-ol, α-amyrin, $\Delta^{5,24(25)}$-stigmastadienol, lupeol, gramisterol, Δ^7-sitosterol, cycloartenol, cycloeucalenol, Δ^7-avenasterol, 28-methylobtusifoliol, 24-methylenecycloartanol, erythrodiol, citrostadienol, uvaol, and oleanol aldehyde. Sitosterol and Δ^5-avenasterol were, quantitatively, the most important phytosterols. Total sterols and most individual sterols differed significantly ($P \geqslant 0.05$) among all three extraction methods with supercritical carbon dioxide extracting the highest total sterol levels (1640 mg/100 g oil) and cold pressed the lowest levels (879 mg/100 g oil).
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Keywords: Phytosterols; Sea buckthorn; Extraction

1. Introduction

Sea Buckthorn is a deciduous shrub which bears yellow or orange berries and has been used for food in both Europe and Asia for centuries. More recently, with the application of modern analytical techniques, the fruit has been shown to have a unique composition emphasizing its potential as a dietary and medicinal supplement (Beveridge, Li, Oomah, & Smith, 1999), and has become noted for its generally high levels of nutritionally and medicinally important components. Phytosterols, as natural components of vegetable oils, have received particular attention because of their capability to lower serum cholesterol levels in humans (Hicks & Moreau, 2001; Jones, MacDougall, Ntanios, & Vanstone, 1997) resulting in significant reduction in the risk of heart disease. Their inclusion in commercial products designed for this purpose, such as the margarine Benecol® (Mellentin, 2005), emphasizes this value. However, phytosterols are also considered to have anti-inflammatory, anti-bacterial, anti-ulcerative and anti-tumor properties (Beveridge, Li, & Drover, 2002) so their contribution to the value of sea buckthorn containing products as a medicinal nutraceutical is clear. Data on the content of individual phytosterols in sea buckthorn are scarce. The solvent extracted seed oil from cultivar sinensis contained 1441 ± 52 mg/kg seed of total phytosterols (Yang, Karlsson, Oksman, & Kallio, 2001). Unfortunately, the oil content of the seed used for these measurements was not reported, but if the oil content is assumed to average 14.2% (Beveridge et al., 1999) then this total phytosterol value represents 1015 mg/100 g oil. Quantitatively, the most important phytosterol present in sea buckthorn seed (sinensis) was sitosterol with a mixture of Δ^5-avenasterol and obtusifoliol providing a second fraction of lesser quantitative importance.

Traditionally, oils were extracted from seed substrates by pressure applied cold (cold pressing extraction, CPE) and more recently organic solvents such as hexane (HE) have been used because they are more efficient and provide higher oil yields. The use of supercritical carbon dioxide (SCE) for extraction of oils destined for nutraceutical

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applications has become desirable because removal of the extracting solvent is complete as residual carbon dioxide volatilizes on exposure to atmosphere. In this study, the effects of these three major extraction methods on the quantities and identity of phytosterols in sea buckthorn seed oil has been determined through careful application of GC–MS methodologies (Beveridge et al., 2002).

2. Materials and methods

All solvents were of reagent grade and were used without any further purification. Hexane was a commercial mixture of glass distilled hexanes (Caledon Laboratories, Georgetown, Ont., Canada). Aluminum-backed, precoated silica gel 60F thin-layer chromatography (TLC) plates, 0.25 mm layer thickness, were obtained from Whatman Ltd. (Maidstone, Kent, UK). Potassium hydroxide pellets (85% purity) were purchased from BDH Chemicals Inc. (Poole, UK). Reference phytosterols, α-amyrin, 5α-cholestane (internal standard), fucosterol, lupeol, sitostanol, β-sitosterol, and uvaol were from Sigma–Aldrich Canada Ltd. (Oakville, Ont., Canada). β-Amyrin, cycloartenol, and erythrodiol were from Extrasynthese S. A. (Genay, France). Lanosterol was from ICN Biomedicals Inc. (Aurora, OH, USA), and a plant sterol mixture containing cholesterol, brassicasterol, campesterol, stigmasterol, and sitosterol in chloroform was purchased from Matreya Inc. (Pleasant Gap, PA, USA). Tri-Sil® reagent (Pierce Chemical Co., Rockford, IL, USA) was used for the preparation of TMS (trimethylsilyl) ether derivatives. Methods and procedures applied were essentially those employed earlier (Beveridge et al., 2002).

2.1. Seeds and oils

Sea buckthorn seeds (cv. Indian Summer) retained on 1.6 mm screens on passage through a standard fruit finisher (F.H. Langsendamp Co. Inc., Indianapolis, IN, USA) were washed and air dried at room temperature (∼20 °C). Cold pressed oils were expressed at 10,000 psi in a Carver laboratory press (Fred S. Carver Inc,. Summit, NJ, USA). For HE and SCE extraction, seeds were ground to pass 1 mm screen in a Wiley mill. HE was performed by stirring for 2 h at ambient temperature (∼20 °C) in hexane (1:3, w:v) and solvent removed by vacuum filtration through Whatman #5 paper. This extraction was repeated once for a 2 h extraction period followed by a repeat for 1 h extraction. The three extracts were pooled and solvent removed by rotatory evaporation at 30 °C. The resulting oil was transferred to an amber bottle and the last traces of solvent removed with a nitrogen purge. SCE was performed with a Hypex Technologies (Ganges, BC, Canada) super critical fluid extractor. Ground seed was placed in a 1000 mL extraction chamber in the approximate geometric center. The void volume at either end of the chamber was filled with glass beads and glass wool to stabilize the extraction bed. Extraction was carried out at 51.7 MPa, 50 °C, at a CO_2 flow rate of 5.87 kg/h. All extracted oils were stored in amber bottles, purged with nitrogen, at −18 °C.

2.2. Preparation of unsaponifiable fraction

One millilitre of 0.1% (w/v) 5α-cholestane (internal standard) in *tert*-butyl methyl ether was added to 1 g of oil in a 100 mL stoppered flask together with KOH (20 ml, 1 M) in methanol and stirred overnight. This solution was mixed with 40 mL of distilled water and extracted three times with 30 mL of *tert*-butyl methyl ether. The combined organic extract was washed with 15 mL of 0.5 M KOH in methanol, followed by repeated 30 mL distilled water washes, followed by one washing with 15 mL of saturated sodium chloride. The solvents were removed by rotary vacuum evaporation at 30 °C, and the residue was left under continuous vacuum overnight to remove solvent traces. Preparations were done in duplicate and stored at −20 °C, in the dark, until further analysis. Saponification progress was monitored using TLC plates developed in hexane/ethyl ether/glacial acetic acid (80:20:1, v/v/v). Plates were viewed under UV light (254 and 366 nm), then sprayed with 10% ethanolic phosphomolybdic acid, and charred.

2.3. Phytosterol derivatization

TMS ether derivatives of the sterols were prepared based on the method reported by Beveridge et al. (2002). For identification purposes residue dilution was to 0.1–0.3 mL followed by gas chromatography–mass spectrometry (GC–MS).

2.4. Phytosterol GC–FID and GC–MS conditions

Analysis of the sterol TMS ether derivatives was performed using a DB-5 fused silica capillary column (60 m × 0.32 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA) in an HP 5890A gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with an FID detector as described by Beveridge et al. (2002). Samples (1 μl) were injected using an HP 7673 autosampler and a split–splitless injector with a split ratio of 1:15. The oven temperature was isothermal at 275 °C. The injector and detector temperatures were 280 and 300 °C, respectively, and helium was used as carrier gas at an inlet pressure of 175 kPa, giving a column flow of 1 ml/min. Data were collected by HP 3365 Series II ChemStation (version A.03.34) software.

All of the TMS ether derivatives were run in triplicate. The level of each sterol was calculated as milligrams per 100 g of oil using the following formula; amount = 100-$(PA_s)(m_{is})/(PA_{is})(m)$, where PA_s = sterol peak area, PA_{is} = internal standard peak area, m_{is} = weight (mg) of the internal standard, and m = weight (g) of oil taken for analysis (Beveridge et al., 2002; Reina, White, & Jahngen, 1997). Data were subjected to analysis of variance using the SAS GLM procedure (SAS Institute, 1990).

GC–MS analysis was performed using a Hewlett-Packard 5890A gas chromatograph equipped with a split–splitless injector, a Hewlett-Packard 5970 mass selective detector (MSD), and an HP 7673A autosampler. The split flow was 15 ml/min. The transfer line temperature from GC to MSD was set at 300 °C. The injector, oven, column, carrier gas, column inlet pressure, and injection volume were as described for the GC–FID instrument above.

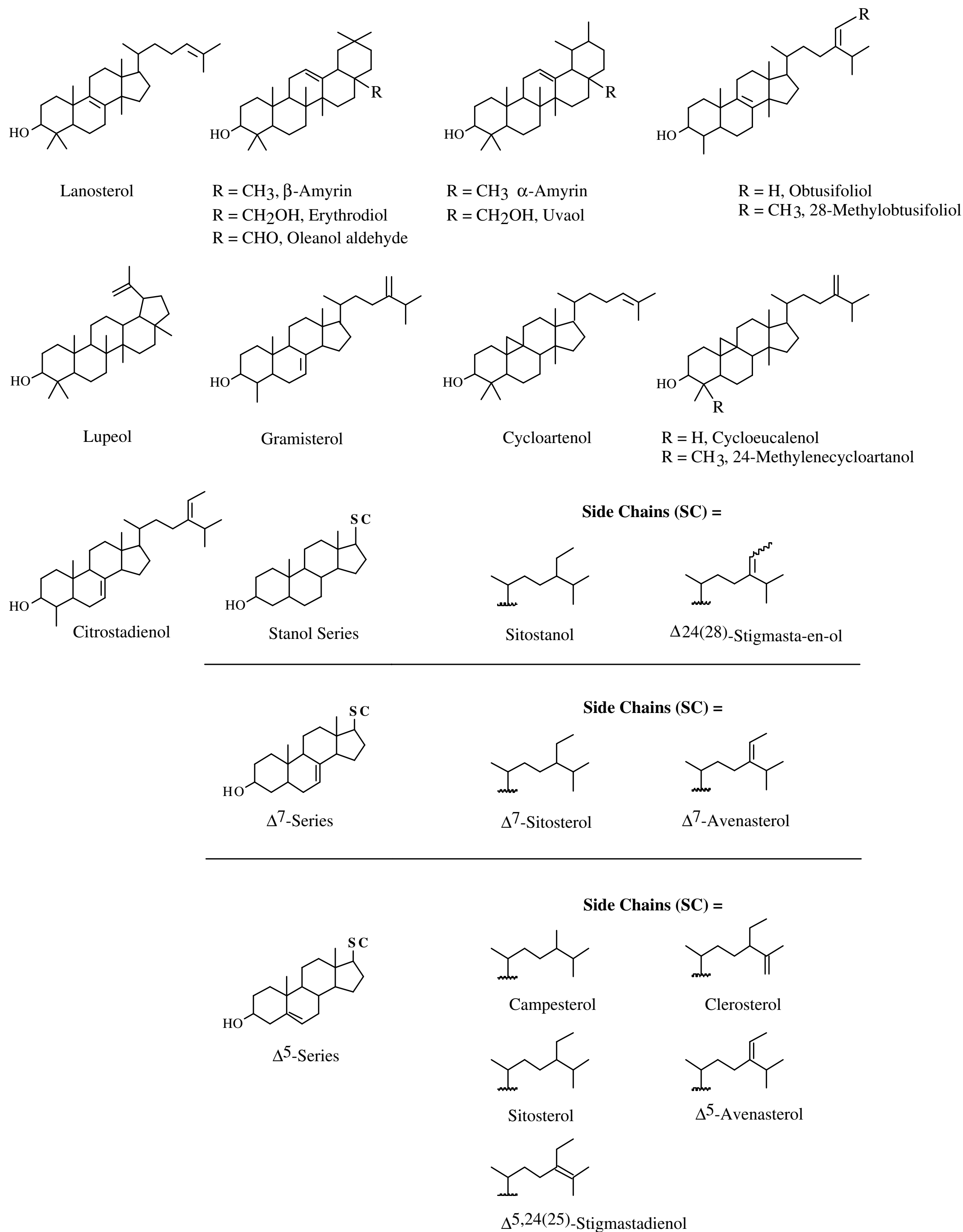

Fig. 1. Structural formulas of phytosterols in sea buckthorn oil.

MSD parameters were as follows: scan mode, 50–600 m/z; threshold, 400; sample rate, 1.1 scans/s; ionizing voltage, 70 eV; and EM voltage, 2000 V. Spectra were acquired by an HP Chemstation software package (G1034C) and peak assignments were carried out by comparing the mass fragmentation patterns and relative retention times with those of reference compounds, literature sources, and the Wiley MS database. Structures are shown in Fig. 1.

3. Results and discussion

Chromatographic analysis of the unsaponifiable fraction of sea buckthorn seed oil showed 17 identifiable phytosterol peaks labelled A through Q (Fig. 2, Table 1). The identities of campesterol, lanosterol, sitosterol, β-amyrin, sitostanol, α-amyrin, lupeol, cycloartenol, erythrodiol and uvaol were confirmed by comparing their RR_t values and their MS fragments with those of TMS ether derivatives prepared from authentic samples. The sterol TMS ether derivatives of commercial olive (Filippo Berio Co. Ltd., Viareggio, Lucca, Italy), pumpkin (Omega Nutrition Inc., Vancouver, BC, Canada), safflower (Sunfresh Ltd., Toronto, Ont., Canada), and sunflower (Hain Food Group Inc., Uniondale, NY) oils were prepared as described above. Comparing the results of the GC–MS and GC–FID analyses of the sterol TMS derivatives of these oils with literature values for composition (Beveridge et al., 2002) and references therein allowed us to locate and identify Δ^5-avenasterol, gramisterol, Δ^7-sitosterol, Δ^7-avenasterol, cycloeucalenol 24-methylenecycloartanol, and citrostadienol. As shown by selected ion monitoring, the leading shoulder of peak C contains a trace of lanosterol, peak E a trace of obtusifoliol, peak G has a trace of a stigmastadienol on the leading edge and a trace of $\Delta^{5,24(25)}$-stigmastadienol on the trailing edge. Peak I has a trace of Δ^7-sitosterol on the leading edge.

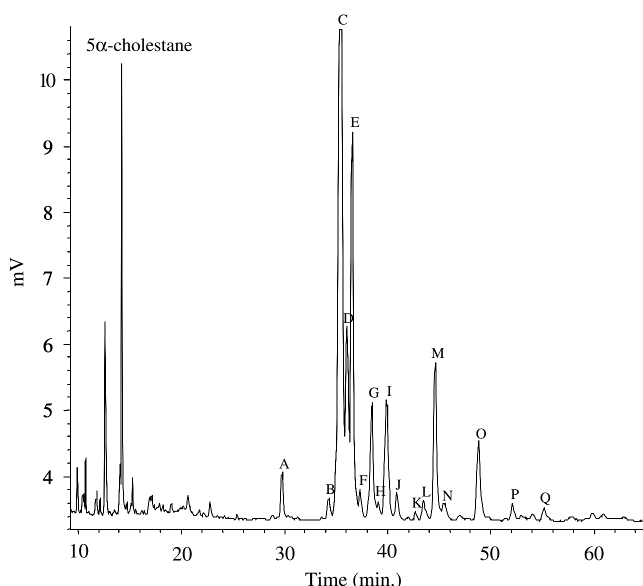

Fig. 2. Gas chromatogram of the FID response of TMS derivatives of sea buckthorn seed oil unsaponifiables. Peaks are identified as in Table 2.

Peak B was tentatively identified as clerosterol based on RR_t evidence (Beveridge et al., 2002; de Blas & del Valle González, 1996) and MS data of the TMS derivative (Beveridge et al., 2002; Farines, Cocallemen, & Soulier, 1988; Goad, 1991).

Peak D consists of two components, β-amyrin and sitostanol, the C 5 saturated homolog of sitosterol, in approximately equal amounts. Adjusting the GC oven temperature to 300 °C allowed separation of sitostanol from the β-amyrin but coalescence of sitostanol with peak F and coalescence of β-amyrin with peak E. Careful assessment of the data from runs at both temperatures suggests that the ratio of β-amyrin to sitostanol is approximately 1.4:1.

There are two compounds in peak E, one major component and one in trace quantities. The position of the minor peak in both the 275 and 300 °C runs does not allow for isolation of a good ion chromatogram but the ratio of ions at m/z 498 and 483 is consistent with the assignment by Yang et al. (2001) of this minor component as obtusifoliol.

The appearance of a molecular ion at m/z 486, and a prominent ion at m/z 388 due to allylic cleavage at C22-23 (Rahier & Benveniste, 1989), are consistent with peak F being the C5 saturated derivative of Δ^5-avenasterol ($\Delta^{24(28)}$-stigmasta-en-ol). Saturation at C5 of 4-desmethyl sterols increases the RR_t (Abidi, 2001). There is an increase of 0.02 U in the RR_t between sitosterol and its C5 saturated derivative sitostanol. There is an identical RR_t increase between Δ^5-avenasterol and peak F. The C5 hydrogenated derivative of Δ^5-avenasterol ($\Delta^{24(28)}$-stigmasta-en-ol) has been observed in the red alga *Gigartina skottsbergii* (Palmero, Seldes, & Gros, 1984) and nematodes (Chitwood & Lusby, 1991) and our data was consistent with the RR_t and MS data reported for the free sterol (Palmero et al., 1984) and the acetate derivative (Chitwood & Lusby, 1991). Peak F is assigned as $\Delta^{24(28)}$-stigmasta-en-ol.

The major component in peak G is α-amyrin. A compound with a molecular ion of 484 m/z on the leading edge of the main α-amyrin peak and another one with the same parent ion on the tailing edge were observed. These peaks were resolved from the main α-amyrin peak at 300 °C and both compounds have fragmentation patterns consistent with a stigmastadienol structure. The leading edge compound has some similarities to $\Delta^{7,25(27)}$-stigmastadienol observed in commercial pumpkin seed oil but the ion at 343 m/z was not as intense as was observed in pumpkin seed oil. The tailing 484 peak has the same pattern and RR_t as $\Delta^{5,24(25)}$-stigmastadienol observed in ginseng oils (Beveridge et al., 2002), oat kernels (Kamal-Eldin, Määttä, Toivo, Lampi, & Piironen, 1998) and sea buckthorn seed oil (Yang et al., 2001). Estimates of the relative quantities of the compounds suggest that the leading edge and tailing compounds comprised 17% and 19% of peak G, respectively.

Table 1
Relative retention times and fragmentation ions used in the identification of the phytosterols in sea buckthorn seed oils

Compound	RR_t[a]	Peak	Main fragmentation ions, *m/e* (RI)[b]					
			M^+	M−15	M−90	M−105	M−129	Others
Campesterol	0.84	A	472(18)	457(6)	382(47)	367(23)	343(58)	315(1), 261(12), 255(18), 227(2), 213(13), 129[c]
Clerosterol	0.97	B	484(24)	469(1)	394(28)	379(16)	355(22)	386(1), 296(2), 255(14), 213(19), 129(94), 55[c]
Lanosterol (tent[d]) tr	0.99	C	498(46)	483(46)		393[c]		187(4), 135(13), 83(11)
Sitosterol	1	C	486(16)	471(6)	396(44)	381(22)	357(54)	255(17), 228(3), 213(12), 129[c]
β-Amyrin	1.02	D	498(1)	483(1)	408 tr	393 tr		279(4), 218[c], 203(40), 190(18), 189(19)
Sitostanol	1.02	D	488(25)	473(30)	398(15)	383(30)	359(2)	431(5), 306(24), 305(27), 290(11), 230(19), 215[c], 75(87)
Δ^5-Avenasterol	1.03	E	484(4)	469(4)	394(3)	379(5)	355(6)	386(65), 371(13), 281(45), 257(27), 255(15), 253(14), 213(17), 211(21), 129(78), 73(81), 55[c]
$\Delta^{24(28)}$-Stigmastaenol	1.05	F	486(1)	471(5)		381 tr		388(77), 373(16), 345(7), 305(12), 283(17), 255(12), 229(16), 216(33), 215(33), 75[c]
A stigmastadienol (tent[d])	1.07	G	484(27)	469(17)		379(18)		386(9), 343(13), 255(11), 253(15), 213(45), 211(12), 129(32), 55[c]
α-Amyrin	1.08	G	498(2)	483(1)	408 (1)	393 (2)		279(6), 218[c], 203(19), 190(21), 189(29)
$\Delta^{5,24(25)}$-Stigmastadienol	1.09	G	484(8)	469(5)	394(6)	379(7)	355(7)	386(31), 371(8), 343(9), 296(28), 281(19), 257(13), 255(11), 253(12), 213(14), 211(12), 129(59), 55[c]
Lupeol + gramisterol (tent[d])	1.10	H						498(1), 484(1), 369(2), 357(28), 279(2), 269(7), 267(2), 257 (2), 241(6), 227(9), 218(52),203(35),189(62), 75[c]
Δ^7-Sitosterol (tr)	1.12	I	486(17)					303(1), 255(41), 229(5), 213(4)
Cycloartenol	1.12	I	498(1)	483(4)	408(31)	393(34)		365(25), 339(16), 325(1), 297(4), 286(8), 271(7), 189(15), 69[c]
Cycloeucalenol	1.13	I		483(3)	408(76)	393(72)		365(12), 353(9), 339(5), 325(4), 300(5), 297(2), 286(3), 283(12), 269(8), 217(13), 189(28), 55[c]
Δ^7-Avenasterol	1.16	J		469(3)		379(1)		386(39), 371(6), 343[c], 296(6), 281(12), 253(26), 213(24)
Unknown	1.21	K				407(27)		385(7), 295(4), 255(3), 213(2), 189(23), 173(52), 73[c], 69(65)
28-Methylobtusifoliol	1.23	L	512(11)	497(25)		407(35)		309(15), 295(4), 281(12), 255(2), 227(20), 215(16), 213(15), 202(32), 69(66), 55[c]
24-Methylenecycloartanol	1.26	M		497(3)	422(30)	407(34)		379(39), 353(13), 339(2), 323(3), 300(9), 297(8), 269(9), 255(5), 241(6), 73[c], 69(89)
Unknown	1.28	N	498(26)	483(8)		408(4)		393(16), 295(5), 281(8), 269(7), 243(17), 241(17), 227(33), 73(73), 55[c]
Erythrodiol	1.37	O			496(33)	481(1)		391(3), 279(1), 216[c], 203(40)
Citrostadienol	1.38	O	498(1)	483(6)	408(1)	393(5)		400(48), 393(5), 385(6), 357[c], 310(8), 295(12), 267(24), 241(14), 227(18)
Uvaol	1.50	P			496(52)	481 tr		391 tr, 281(6), 216(65), 203(79), 202(22), 201(26), 73[c]
Oleanol aldehyde (tent[d])	1.56	Q						279(6), 232(29), 203[c], 190(45), 189(32), 175(17), 133(12)

[a] Retention times relative to sitosterol TMS ether (35.70 min) using a DB5 60 m column.
[b] Intensity relative to base peak (%).
[c] Base peak (relative intensity = 100%). The internal standard 5α-cholestane elutes with a relative retention time of 0.40.
[d] Tent are tentative assignments. tr = trace.

Peak H is small and does not provide a substantial number of ions in the mass spectra. It appears to consist of two co-eluting compounds with molecular ions of 498 and 484 m/z. The RR_t and MS patterns suggests peak H is a mixture of lupeol and gramisterol (Conte, Frega, & Capella, 1983; Chryssafidis, Maggos, Kiosseoglou, & Boskou, 1992; Esuoso, Lutz, Bayer, & Kutubuddin, 2000; Itoh, Tamura, & Matsumoto, 1973; Homberg & Bielefeld, 1990; Kamal-Eldin, Appelqvist, Yousif, & Iskander, 1992).

Using selected ion monitoring, peak I reveals three components centered at $RR_t = 1.116$, 1.119 and 1.125, respectively. The identity of the minor component on the leading edge ($RR_t = 1.116$) was assigned as Δ^7-sitosterol based on the RR_t and MS of a known compound present in ginseng oil (Beveridge et al., 2002), and is collaborated by literature data (Goad, 1991; Dumazer, Farines, & Soulier, 1986; Rahier & Benveniste, 1989). The compound centered at $RR_t = 1.119$ is cycloartenol based on RR_t and MS data of authentic compounds supported by literature data (Goad, 1991; Kamal-Eldin et al., 1992; Yang et al., 2001). The compound centered at $RR_t = 1.125$ is tentatively identified as cycloeucalenol based on literature RR_t and MS data (Goad, 1991; Kamal-Eldin et al., 1992; Yang et al., 2001). The two major components in peak I are cycloartenol and probably cycloeucalenol in approximately equal amounts.

Peak K is tentatively identified as a Δ^7-sterol based on agreement of the RR_t and MS data with literature

(Kamal-Eldin et al., 1992). The identity of Peak L was assigned as 28-methylobtusifoliol based on agreement of RR_t and MS data with literature (Ge, 1992; Yang et al., 2001). Peak N appears to be the same unidentified compound previously reported (Yang et al., 2001) and observed to elute immediately following 24-methylenecycloartanol (24-MCA).

Peak O is comprised of two components, erythrodiol and citrostadienol. Using selected ion monitoring, erythrodiol was shown to elute slightly before the citrostadienol. The second portion of the peak has the same RR_t and MS profiles as citrostadienol seen in sea buckthorn seed, ginseng, eggplant, sesame, safflower, sunflower, and olive oils (Beveridge et al., 2002; Chryssafidis et al., 1992; Farines et al., 1988; Homberg & Bielefeld, 1990; Kamal-Eldin et al., 1992; Yang et al., 2001).

Peak Q is tentatively identified as oleanol aldehyde. The amount of compound present in this peak is small with only the major ions observed in the MS spectrum. The compound is not oleanolic or ursolic acid due to the lack of major ions at 482 and 320 (Burnouf-Radosevich, Delfel, & England, 1985). The ratio of the 232 and 203 ions present in the spectrum reported here agrees with the ratio provided for oleanol aldehyde (Salenko, Kukina, Karamyshev, Sidel'nikov, & Pentegova, 1986).

In summary, eight compounds have been identified that have not been previously reported in sea buckthorn seed oil. These include clerosterol, (peak B), a trace of lanosterol (under or just slightly ahead of peak C), $\Delta^{24(28)}$-stigmastaenol (peak F), lupeol and gramisterol (peak H), erythrodiol (peak O), uvaol (peak P) and oleanol aldehyde (Peak Q). Of this group, lupeol, erythrodiol, uvaol and oleanol aldehyde have been observed in sea buckthorn leaves (Gonchareva & Glushenkova, 1995; Salenko et al., 1986) and pulp oil (Glazunova, Gachechiladze, & Mukhtarova, 1989; Salenko, Sidel'nikov, Troshkov, Raldugin, & Pentegova, 1982; Xin, Li, Wu, Sun, & Aitztmuller, 1995). Lanosterol has been observed in sea buckthorn pulp oil (Ge, 1992; Wang, Ge, & Zhi, 1989).

As shown in Table 2, the amount of individual phytosterol components can vary significantly depending on the extraction method. Supercritical fluid extraction results in the highest amount of total phytosterols followed by hexane and cold press extractions, 1640, 1326, and 879 mg/100 g oil, respectively, and is similar to results obtained in grape seed extractions (Beveridge, Girard, Kopp, & Drover, 2005). The same trend is also observed for individual phytosterols. One previous report (Xin et al., 1995) suggests that there were no differences in yield of total and individual sterols whether extracted with supercritical carbon dioxide or hexane. Probably some of the differences observed here result from differential extractions of phytosterols as their individual solubilities in hexane, supercritical carbon dioxide and sea buckthorn oil almost certainly vary. Still the supercritical carbon dioxide provides for extraction of phytosterols at least equal to but probably in excess of that provided for by hexane. This is important since the solvent residue free supercritical carbon dioxide extracted oil preferred for nutraceutical applications, will also provide a full measure of the phytosterols present. The total quantity of phytosterols in both supercritical and hexane extracted oils is quite high exceeding 1 g per 100 g oil. Sitosterol is quantatively the most important phytosterol representing 48%, 51%, and 53% of the phytosterols in supercritical carbon dioxide, hexane and cold pressed oil, respectively. These levels of both total phytosterol and sitosterol are among the highest levels reported for seed oils.

Table 2
Phytosterols contents (mg/100 g oil) in sea buckthorn seed oil from supercritical fluid (SFE), hexane (HE), and cold press (CPE) extractions

Peak	Phytosterol component	Extraction method		
		SFE	HE	CPE
A	Campesterol	$24.0^{a,*}$	20.6^{b}	13.9^{c}
B	Clerosterol	14.3^{a}	11.9^{b}	8.4^{c}
C	Lanosterol (tr) + sitosterol	787.4^{a}	673.0^{b}	462.2^{c}
D	β-Amyrin + sitostanol	122.5^{a}	94.3^{b}	69.5^{c}
E	Δ^{5}-Avenasterol + obtusifoliol (tr)	218.5^{a}	165.9^{b}	97.3^{c}
F	$\Delta^{(24,28)}$-Stigmasta-en-ol	19.6^{a}	12.9^{b}	7.2^{c}
G	A stigmastadienol + α-amyrin + $\Delta^{5,24(25)}$-stigmastadienol	81.4^{a}	73.5^{b}	56.7^{c}
H	Gramisterol + lupeol	11.5^{a}	9.0^{b}	6.8^{c}
I	Δ^{7}-Sitosterol (tr) + cycloartenol + cycloeucalenol	102.3^{a}	74.0^{b}	41.1^{c}
J	Δ^{7}-Avenasterol	17.7^{a}	14.2^{b}	9.2^{c}
K	Unknown	5.4^{a}	4.2^{b}	3.6^{b}
L	28-Methylobtusifoliol	16.6^{a}	13.6^{b}	12.0^{c}
M	24-Methylenecycloartanol	105.8^{a}	76.1^{b}	37.7^{c}
N	Unknown	17.4^{a}	14.1^{b}	10.9^{c}
O	Erythrodiol + citrostadienol	67.5^{a}	53.1^{b}	29.9^{c}
P	Uvaol	13.6^{a}	9.3^{b}	7.3^{c}
Q	Oleanol aldehyde	14.9^{a}	6.8^{b}	5.7^{b}
Total		1640.4^{a}	1326.5^{b}	879.4^{c}

* Values in each row with a common letter are not significantly different ($P = 0.05$) according to Duncan's New multiple range test.

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