JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Extraction and Purification of Squalene from *Amaranthus* Grain

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Grain amaranth has been suggested as an alternative to marine animals as a natural source of squalene. Oil contents, squalene contents, and fatty acid profiles were determined in 11 genotypes of four grain amaranth species. Although the oil contents of grain amaranth were low (from 5.1% in *Amaranthus tricolor* to 7.7% in *Amaranthus cruentus*) as compared to other oil-containing grains, high concentrations of squalene were found in total lipids, ranging from 3.6% in *Amaranthus hypochondriacus* to 6.1% in *A. tricolor*. The major fatty acids in *Amaranthus* oil consisted of palmitic acid (19.1−23.4%), oleic acid (18.7−38.9%), and linoleic acid (36.7−55.9%). A high degree of unsaturation was observed in *Amaranthus* oils, with S/U ratios of 0.26−0.32. A method to isolate and purify the squalene from *Amaranthus* oil was developed. After the saponification of K112, the squalene content increased from 4.2% in the crude oil to 43.3% in the unsaponifiables by the removal of the saponifiables. The unsaponifiables were fractionated by silica gel column chromatography to get highly purified squalene. The squalene purity in certain fractions was as high as 98%. Combining the fractions rich in squalene gave a 94% squalene concentrate, with a yield of 90%. The structure of squalene in the purified sample was confirmed by comparison of its ultraviolet spectrum with a standard and from its nuclear magnetic resonance spectra.

KEYWORDS: *Amaranthus*; squalene; oil contents; fatty acid; purification; NMR spectra

INTRODUCTION

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is a biosynthetic precursor to all steroids. It is an important ingredient in skin cosmetics due to its photoprotective role and as a lubricant for computer disks due to its thermostability (*1*, *2*). It is hypothesized that the decreased risk for various cancers associated with high olive oil consumption could be due to the presence of squalene (*3*). It has also been suggested that squalene has a chemopreventive effect on colon cancer (*4*). Other beneficial effects on health can be attributed to its hypocholesterolaemic action, in combination with the administration of tocotrienols (*5*). Additionally, the use of squalene alone has been demonstrated to be effective in decreasing serum cholesterol levels (*6*).

The traditional source for squalene is primarily from shark (*Centrophorus squamosus*) and whale (*Physeter macrocephalus*) liver oil (*7*). The use of squalene in cosmetic applications is limited by the uncertainty of its availability as a result of international concern for the protection of marine animals. In addition, the presence of similar compounds, such as cholesterol, in the oils from marine animal liver can make squalene purification difficult. There is interest in other potential sources of squalene, and plant sources are being widely prospected. Squalene contents in olive, wheat germ, and rice bran oils are in the 0.1−0.7% range (*8*), which is not high enough for them to be considered as a viable resource. It was reported that squalene was present in up to 0.46% of the leaf dry weight of Macaronesian *Echium* plants (*9*) and that squalene was found in minute amounts in all European *Echium* species. *Amaranthus* grain is potentially an important source of squalene. *Amaranthus* oil has been reported to contain larger amounts of squalene (2.4−8.0%) than other common vegetable oils (*10*, *11*). Squalene content in *Amaranthus cruentus* has been reported to be 0.43% of the total seed weight (*9*). In addition, squalene was present at 0.73% in the seed of *Amaranthus hypochondriacus* and 1.32% of *Amaranthus pumilus* (*12*).

Amaranthus (Amaranthaceae) includes over 75 wild and weedy species native to tropical and temperate regions of the whole world but is most diverse in the Americas (*13*). Presently, cultivated grain is grown in many areas of the world, including Central and South America, Africa, India, China, and the United States. In this study, we investigated the oil content, the squalene content, and the fatty acid profile of 11 genotypes of grain amaranth cultivated in China. Pharmaceutical and industrial applications usually require high concentrations of squalene, so a method for squalene purification was also developed.

MATERIALS AND METHODS

Materials. Plant material consisted of 11 genotypes of four *Amaranthus* species, which were grown in the same environment at the experimental farm of Hubei Academy of Agricultural Science,

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10.1021/jf010918p CCC: $22.00
Published on Web 12/19/2001

Table 1. Oil and Squalene Contents in Grains of 11 *Amaranthus* Genotypes

genotypes	oil[a] (%)	squalene[b] (%)	seed color[c]	1000 weight (g)	origin of genotype
			A. cruentus		
Cr024	7.41	4.70	++++	0.41	Nigeria
Cr048	6.40	4.52	+	0.68	Mexico
Czech	6.45	5.04	++++	0.55	Czechoslovakia
K112	6.75	4.20	+++	0.70	U.S.
R104	6.60	5.44	++	0.68	U.S.
V69	7.72	4.63	+++	0.67	China
K350	5.57	5.14	+++	0.83	China
			A. hybridus		
Hr027	6.40	5.23	++++	0.34	Zambia
			A. hypochondriacus		
$HyNO_2$	7.05	5.01	+++	0.76	U.S.
$HyNO_3$	5.35	3.62	++++	0.57	U.S.
			A. tricolor		
Tr017	5.08	6.14	+++++	0.58	China
overall mean	6.43	4.88		0.62	
LSD ($P < 0.05$)	0.51	0.64			

[a] Oil contents based on dry matter were the means of triplicate determination. [b] Squalene contents in *Amaranthus* oils were the means of triplicate determination. [c] Seed color was shown qualitatively by "+". "+++++" indicates the black color, and + indicates the white color. The number of the + indicates the shade of the color.

Wuhan, China. These genotypes originated from six countries (Table 1) and were provided by the USDA-ARS National Plant Germplasm Collection, held at the Plant Introduction Station, Iowa State University, Ames, Iowa, and the Chinese Academy of Agricultural Sciences, Beijing, China (Table 1). Squalene (99.9% purity) standard was from Sigma Chemical Co. (St. Louis, MO).

Oil Extraction. The grain was ground to flour with a laboratory mill (Kenwood) for oil extraction. Samples (30 g) were milled at the fifth speed for 1.0 min time periods with 1.0 min rest after each. Different total milling times, 0, 1, 2, and 5 min, respectively, were used to examine the effect of milling on the particle size and the oil extraction. The size distribution of samples with different milling times was measured by sieve analysis. The powdered samples were extracted for oils by an extraction/desolventizing unit, Soxtec System HT6 (Tecator, Sweden), with petroleum ether (boiling range 40−60 °C) containing 0.01% butylated hydroxytoluene as an antioxidant to avoid the possible deterioration of unsaturated fatty acids. Furthermore, a chloroform−methanol−water mixture solvent system, widely used for lipid extraction from natural tissue (*14*), was also used for oil extraction to compare the effect of various methods of oil extraction.

Isolation of the Unsaponifiables. The extracted *Amaranthus* oil was used for squalene isolation and purification by two steps. The first step was to remove the saponifiables. *Amaranthus* oil (3 g) was dissolved in a mixture of 30 mL of 95% ethanol and 5 mL of 50% KOH and refluxed for 1 h. The reaction mixture was transferred to an extraction cylinder and washed to 40 mL with 95% ethanol and then diluted to 80 mL with distilled water. The unsaponifiables were extracted five times, using 50 mL of petroleum ether each time. The combined extracts were washed using 25 mL portions of 10% ethanol in water until the wash solution no longer gave a pink color after the addition of phenolphthalein solution (1% in ethanol, wt/wt). The petroleum ether layer was dried by anhydrous sodium sulfate. The solution was filtered, and the solvent was removed in a rotary evaporator. The unsaponifiables fraction in the oil was then expressed in weight percent.

Column Chromatography. The unsaponifiables were further purified for squalene by column chromatography on a silica gel (24 g, 70−230 mesh, Sigma Co.) column. A solution of 0.18 g of unsaponifiables in 5 mL of petroleum ether was loaded and eluted by washing the column with 1% diethyl ether in petroleum ether, at a flow rate of approximately 1.0 mL/min. Test tubes (10 mL with screw cap) were used for fraction collecting. Thin-layer chromatography (TLC) was used for detection. Squalene appeared completely in the 1% diethyl ether in petroleum ether eluate. The eluate was evaporated by vacuum evaporation to give colorless squalene liquid. The residues in the column were washed out by chloroform.

Identification of Components in the Fractions. TLC was used to identify the components in the fractions based on their relative mobility value (R_f). The purified samples and Sigma standard squalene (>99%) were spotted directly on the plate. The TLC was carried out on precoated silica gel 60 F_{254} plates (0.25 mm, Merck Co.), and the plates were developed by hexanes−ethyl acetate (6:1, v/v). The spots were visualized by iodine fumes. The R_f value of squalene was 0.9.

Fatty Acid Analysis. The American Oil Chemists' Society (AOCS) official method (*15*) with minor changes was used to prepare the fatty acid methyl esters (FAMEs) for fatty acid analysis. The FAMEs were detected by an HP-6890 gas chromatograph (GC) (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and a Supelco (Bellefonte, PA) Omegawax 250 capillary column (30 m × 0.25 mm i.d.). The GC conditions were as follows: initial oven temperature (170 °C), initial time (15 min), heating rate (2 °C/min), final temperature (220 °C), final holding time (10 min), injection port temperature (280 °C), and detector temperature (260 °C). Peaks were identified by comparison with FAME standards from Sigma. Nonadecanoic acid (19:0) methyl ester was added as an internal standard. The absence of 19:0 in the samples was previously checked before sample analysis.

Squalene Analysis and Structural Identification. Squalene contents in extracted oil samples and purified samples were determined using a high-performance liquid chromatography (HPLC) system (Hewlett-Packard 1100) under the following conditions: RP-C18 column (Nucleosil 100-C18, 5 μm, 250 × 4 mm i.d.); photodiode array detector (214 nm wavelength used for squalene detection); isocratic elution with methanol-2-propanol-acetic acid (91.95:8:0.05, v/v); injection volume, 20 μL; and flow rate, 1.0 mL/min. Squalene was identified by comparing the retention time to pure squalene (>99%) from Sigma. Squalene was eluted at 9.9 min. Samples and Sigma standard were diluted to a final concentration of 20−100 ppm.

UV spectra of purified sample and squalene standard were obtained with a SPECTRONIC GENESYS 5 spectrophotometer (Milton Roy Co., Rochester, NY) to compare their absorbance in the UV range. In addition, the structure of purified squalene was confirmed by ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) spectra. The chemical shifts were presented as parts per million with tetramethylsilane as an internal standard.

RESULTS AND DISCUSSION

Oil Extraction. To extract the oil from grain amaranth, the grains must be ground to flour. The efficiency of oil extraction is usually dependent upon the degree of grinding. The more finely the flour is milled, the higher the oil extraction efficiency. R104, an *Amaranthus* genotype widely cultivated in China, was used to investigate the effect of milling on oil extraction. The grain size is small, typically approximately 1 mm in diameter,

Table 2. Main Fatty Acid Composition of 11 *Amaranthus* Genotypes[a]

	fatty acid (%)						
genotypes	myristic 14:0	palmitic 16:0	stearic 18:0	oleic 18:1	linoleic 18:2	all others	S/U ratio[b]
			A. cruentus				
Cr024	0.2	20.3	0.9	30.4	46.1	2.1	0.28
Cr048	0.3	22.7	0.4	37.8	36.7	2.1	0.31
Czech	0.2	19.5	0.6	36.1	41.3	2.3	0.26
K112	0.3	21.6	Tr	38.9	38.1	1.1	0.28
R104	Tr	21.8	Tr	38.2	39.4	0.6	0.28
V69	0.2	20.3	0.8	30.1	46.4	2.2	0.28
K350	0.3	23.4	0.6	32.3	43.0	0.4	0.32
			A. hybridus				
Hr027	0.3	20.6	1.8	18.7	55.9	0.5	0.29
			A. hypochondriacus				
$HyNO_2$	0.2	19.9	1.1	29.8	47.2	1.8	0.27
$HyNO_3$	0.3	19.1	1.1	24.0	53.8	1.7	0.26
			A. tricolor				
Tr017	0.2	19.5	1.0	27.5	49.9	1.9	0.27
overall mean	0.2	20.8	0.8	31.3	45.3	1.5	0.28

[a] Means of duplicate determinations. [b] S/U ratio = saturated/unsaturated = (14:0 + 16:0 + 18:0)/(18:1 + 18:2).

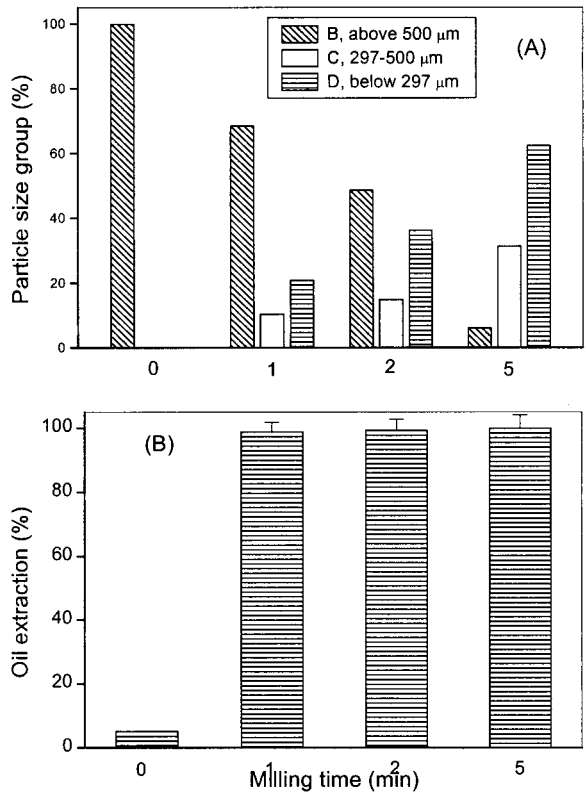

Figure 1. Effect of milling time on flour particle size and oil extraction. (A) Size distribution of ground powder with different milling time. (B) Effect of milling time on oil extraction.

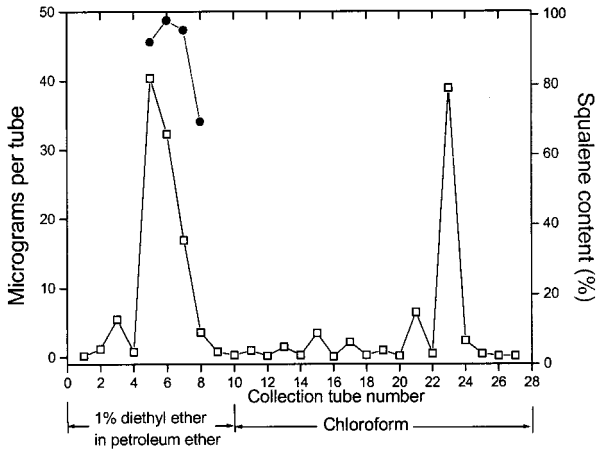

Figure 2. Separation of the unsaponifiables of *Amaranthus* oil by silica gel column. Microgram per tube (□); squalene content (●).

and lenticular-shaped. It was observed that a milling time of 1 min had just split the grain, yielding little fine powder; the size above 500 μm with this milling time was as high as 68.5% (Figure 1A). Increasing the milling time yielded finer powder, with 5 min of milling giving a powder where 93.8% was below 500 μm (62.4% below 297 μm, and 31.4% between 297 and 500 μm).

Soxtec System was used for oil extraction from powdered samples. In a typical extraction process, 5 g powdered samples were first immersed in 40 mL of boiling petroleum ether for 30 min to dissolve most of the soluble material. Second, the samples were raised above the solvent surface to permit an efficient rinsing of the samples for 45 min with solvent from the condensers. Then, the solvent was removed by evaporating to the condensers to give the oils. The difference in the oil extraction from powder milled for 1 min and that directly from unmilled grain is obvious (Figure 1B). The lipid of *A. cruentus* seed is concentrated in the embryonic tissue (*16*). The oil extraction efficiency is so low from unmilled seeds because it is difficult for the solvent to penetrate the seed coat to access the embryo for lipid extraction. No difference in oil extraction was observed with a milling time of 1−5 min (Figure 1B), which meant that 1 min was sufficient. These results also showed that the oil extraction could be carried out efficiently as long as the seed was at least split.

Another extraction method by a solvent mixture (chloroform−methanol−water, 1:2:0.8, v/v), used widely for lipid extraction from natural products and animal tissues, described by Bligh and Dyer (*14*), was also used for crude oil extraction. The mass ratio of solvent/sample was 20:1. Although this method is used widely for extracting lipids from animal, plant, and bacterial tissues, the measured lipid content was not as high as that obtained by Soxtec extraction. This might be due to the low ratio of solvent to sample used in mixed solvents. Another disadvantage of this method for total oil extraction is that it is solvent-consuming and time-consuming to remove contaminants carried in by the mixed solvent.

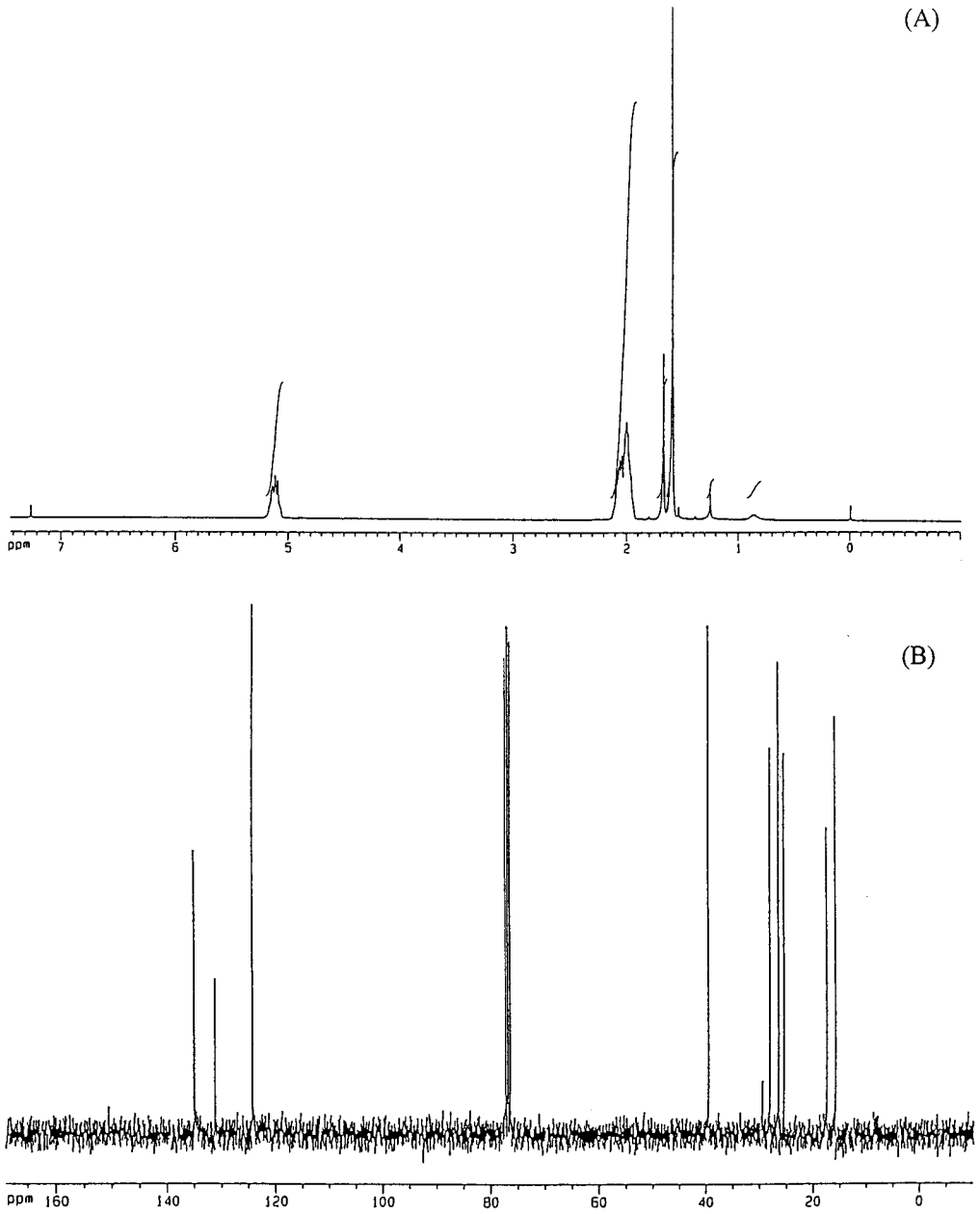

Figure 3. NMR spectrum of purified squalene. (A) ^{1}H NMR (300 MHz, $CDCl_3$) spectrum. (B) ^{13}C NMR (75 MHz, $CDCl_3$) spectrum.

Contents of Oil, Squalene, and Fatty Acids. The extracts from different genotypes were evaporated to give light golden- to brown-colored oils. The crude oil content of 11 *Amaranthus* genotypes ranged from 5.1% in Tr017 to 7.7% in V69, with an average of 6.4% (Table 1). These results are consistent with the report of a total crude fat range from 5.2% (*Amaranthus acutilobus*) to 7.6% (*Aamaranthus dubius*) in a study involving 21 accessions and eight species (*1*). The results showed some significant variation in oil content among various genotypes. *A. cruentus* seemed to have a relatively higher oil content (mean 6.7%) than other species. No obvious relationships were observed between the oil content and the seed color and seed size. Although *Amaranthus* grain was low in oil content as compared to many other oil-containing grains, *Amaranthus* oil contained considerably higher concentrations of squalene. Squalene contents in some important commercial oils are quite low, such as 0.002% in coconut; 0.01% in sunflower and cotton; 0.03% in maize, peanut, and rapeseed; 0.3% in rice bran; and 0.4% in olive oil (*16*). Squalene content in *Amaranthus* seed oils ranged from 3.6% in $HyNO_3$ (*A. hypochondiacus*) to 6.1% in Tr017 (*A. tricolor*), with an average content of 4.9%. Significant differences occurred in the content of squalene among genotypes. No significant correlations were observed between the squalene contents and seed color and seed size. The high content of squalene in grain amaranth may represent a viable new source of squalene. We note that the greatly increased *Amaranthus* grain production in China in recent years may provide the agricultural base for economic production of squalene.

The fatty acid composition reflects the stability and nutritional quality of fats and oils. The major fatty acid components of oils from seeds of 11 *Amaranthus* genotypes were similar (Table 2). The preponderant fatty acids were palmitic acid, oleic acid, and linoleic acid. The remaining fatty acids occurred in much lower amounts (trace to 2%). The results were consistent with previous reports (*7*). For 11 genotypes of four *Amaranthus* species, oleic acid ranged from 18.7 to 38.9%, and linoleic acid ranged from 36.7 to 55.9%, but palmitic acid only ranged from 19.1 to 23.4%. Two genotypes of *A. hypochondriacus* had a

higher level of linoleic acid (mean 50.5%) and a lower level of oleic acid (mean 26.9%) as compared to other species (overall mean: linoleic acid, 45.3%; oleic acid, 31.3%). In addition, *Amaranthus* oils were highly unsaturated. The saturated/unsaturated fatty acid ratios of different genotypes were close, ranging from 0.26 to 0.32.

Isolation and Purification. Pharmaceutical and industrial applications usually require high squalene concentration. A method has been suggested for its recovery from olive oil deodorization distillate, which is a byproduct of olive oil refining, by supercritical carbon dioxide extraction using a 3 m $\times$ 30 mm extraction column (*17*). Under optimized operating conditions, an extracted squalene purity of 90.0% and an extraction yield of 91.1% were obtained. The present study was carried out to obtain higher purity squalene on a laboratory scale from *Amaranthus* oil. Squalene is an unsaponifiable lipid and is not chemically altered by alkali. The first step to isolate the squalene was to remove the saponifiable lipids. After 3.00 g of oil from K112 was saponified, 0.271 g of unsaponifiables was obtained, which represented 9.0% of the total lipids. By removal of the saponifiables, the squalene content increased from 4.2% in crude oil to 43.3% in the unsaponifiables. The unsaponifiable lipid constituents of seed oils contained a variety of substances, including hydrocarbons, tocopherols, sterols, and terpene alcohols. The squalene was purified further by silica gel column chromatography. Squalene was eluted out completely in the 1.0% diethyl ether in petroleum ether fraction (Figure 2). Several fractions rich in squalene, detected by TLC, were collected. The squalene contents in these fractions were analyzed by HPLC. The first two fractions consisted of 76.2 and 93.2% of squalene, respectively. The squalene content in the third fraction was above 98%. The four fractions rich in squalene, detected by TLC with the Sigma standard, were combined, and the solvent was evaporated by rotary evaporation to yield a colorless liquid, with a concentration of 94% and a recovery of 90%. Other unsaponifiable lipids in the column were eluted by chloroform (Figure 2).

The structure of purified squalene was verified by its UV spectra and nuclear magnetic resonance (NMR) spectra. The UV spectra (not shown) of the squalene standard and enriched fraction were compared to determine possible impure compounds with similar physical/chemical properties. The sample with relatively high concentration was used for UV measurement to increase the concentration of potential impurities in the enriched sample. The UV spectrum of the squalene fraction displayed a strong absorbance only at 214 nm in the UV range, well in accord with the standard sample, which indicated that no impurities were observable in the UV range. The structure of the purified squalene was further confirmed by its ^{1}H and ^{13}C NMR spectrascopic data. The ^{1}H NMR (300 MHz, $CDCl_3$) (Figure 3A) showed methyl groups at δ 1.60 (s, 18H) and δ 1.68 (s, 6H), methylene groups at δ 1.99−2.03 (m, 20H), and internal vinylic signals at δ 5.06−5.15 (m, 6H). The ^{13}C NMR (75 MHz, $CDCl_3$) (Figure 3B) showed eight methyl carbons at δ 16.0−25.7, 10 methylene carbons at δ 26.7−39.8, and 12 double bond carbons at δ 124.3−135.1. The NMR spectra were consistent with the published literature (*18*).

Although grain amaranth is not considered a typical oilseed crop, it has been identified as a new crop rich in squalene. The oil content of *Amaranthus* seeds is about 6−7%, but *Amaranthus* oil contains a high content of squalene. As the clinical functions of squalene are better elucidated, the importance of grain amaranth as a source of squalene will increase further. Work to screen genotypes containing a higher content of squalene in a wide range of species and to find efficient large-scale separation methods is in progress.

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Received for review July 16, 2001. Revised manuscript received October 24, 2001. Accepted October 29, 2001. Financial support was received from Hong Kong Research Grants Council awards to M.S. and H.C.

JF010918P