

Squalene in Oils Determined as Squalane by Gas—Liquid Chromatography After Hydrogenation of Methyl Esters¹

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ABSTRACT: Low levels ($\leq 0.1\%$) of squalene were anticipated in oils from the blubber of the harp seal *Phoca groenlandica*. The traditional route of saponification and analytical examination of the total unsaponifiables was unattractive. A method developed for squalene in olive oil, reportedly present in the range of 0.3–0.7%, was based on total conversion of the oil to methyl ester of fatty acids by alkali transesterification, followed by hydrogenation over Adam's catalyst (PtO_2). The analysis of the fully saturated methyl esters and any squalane produced concurrently was by gas–liquid chromatography. This method was satisfactory for the small amounts, 0.03% or less, of squalene in seal oil and is also illustrated for olive oil. A flame-ionization detector excessive response of approximately 25% was observed for all levels of squalene tested. The calculated factor of 1.22 should be applied to the peak area for squalane due to the higher response of hydrocarbons relative to the methyl esters of fatty acids and the system of oil components if reporting as fatty acids.

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KEY WORDS: Gas–liquid chromatography, hydrogenation, olive oil, saturated methyl esters, seal oil, squalane, squalene.

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetra-cosa-hexane) is found in many marine organisms (1,2). The liver oils of certain sharks are major sources (3,4) although the actual biochemistry is not well known (5). The body oil of a North Pacific vertebrate fish *Thaleichthys pacificus* contains up to 10% squalene (6). In the latter analysis direct injection of the body oil into a packed gas–liquid chromatography (GLC) column was a quick and simple way to determine the squalene.

Squalene is a minor but potentially important antioxidant found among the unsaponifiables of virgin olive oil at the level of 300 to 700 mg/100 g oil (7,8). Accordingly many olive oil analyses have been executed by various means, including treatment of the oil with diazomethane to allow direct injection

of the oil for simultaneous determination by GLC of the free fatty acids as methyl esters, and of the squalene (9).

A need to measure low levels of squalene in oil from the harp seal *Phoca groenlandica* led to our considering concentration of squalene in the total unsaponifiables. This approach could be based on American Oil Chemists' Society (AOCS) methods and has been used by Shahidi and Wanasundara (10), but is cumbersome and lengthy. In the olive oil field Lanzón *et al.* (11) used squalane as an internal standard in GLC to determine squalene in the methyl esters of olive oil. De Leonardis *et al.* (7) omitted the squalane and adopted total hydrogenation of the esters and of the squalene, permitting measurement of the resulting squalane in conjunction with GLC of the now saturated fatty acids as methyl esters. This approach appeared simpler and suitable for the low levels expected in seal oil.

MATERIALS AND METHODS

All of the seal oils tested were produced from harp seals. Two samples were recent commercial products, a 1998 production from the Carino Company of Newfoundland and a 1999 sample from Les Poissons Frais des Isles of the Magdalen Islands in the Gulf of St. Lawrence. An additional sample produced in Nova Scotia in 1961 by the firm of K. Karlsen and Co., New Harbour, Nova Scotia, had been sealed in brown glass and stored in the dark since production. Two olive oil samples were a retail sample of Greek origin purchased in Halifax and described as extra virgin quality and a similar bottled commercial oil known to be of Cretan origin, the gift of A. Kiritsakis. Palm oil (unrefined) was also purchased retail in Halifax.

The oil samples were converted to methyl esters by transesterification with alkali in methanol, since squalene is adversely affected by boron trifluoride acid catalysis (3). The methyl esters were analyzed on a 30 m \times 0.25 mm Omegawax-320 column (Supelco, Oakville, Ontario, Canada) in a PerkinElmer AutoSystem gas chromatograph (Norwalk, CT) with helium as the carrier gas. Operating conditions were: inject at 200°C, hold 5 min, program at 3°C/min to 230°C, and hold 15 min. The flame-ionization detector (FID) peak area responses for methyl esters of fatty acids were converted to weight percentage of fatty acids. As part of this process in the automated

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analysis of quantitation of the hydrogenated esters the response factor (12) for 18:4n-3 was applied to squalene as it was more or less neutral (0.9730 relative to the 1.000 of methyl 18:0) for the computer program employed.

The hydrogenation of esters of seal oil or of seal oil spiked with 0.1, 0.5, and 1.0% of squalene was executed by first preparing the esters and then dissolving the esters (10–30 mg) in 30 mL of chloroform in a 100 mL flat-bottomed flask containing a Teflon-coated magnetic spin bar. Approximately 20 mg of Adam's catalyst (PtO_2 ; Fluka AG, Buchs, Switzerland) was added, and the whole was stirred gently under a slow flow of hydrogen for a minimum time of 1 h. The totally hydrogenated esters were recovered carefully, because of the low solubility of the longer chain lengths in solvents, and re-analyzed for all chain lengths and for squalane. The vegetable oils received the same treatment.

RESULTS AND DISCUSSION

Seal oil. The choice of Omegawax-320 for the gas-liquid chromatography of methyl esters of seal oil fatty acids eliminates most chain-length overlaps of the complex mixture of fatty acids present in marine oils, but with our program squalene is usually superimposed on the position frequently occupied by nervonic acid (24:1n-9), which immediately follows docosahexaenoic acid (DHA; 22:6n-3) (13). Figure 1 shows that in raw seal oil with 6–7% of DHA and normal GLC operating conditions no peak is observed for the 24:1n-9 (usually $\leq 0.5\%$ of total fatty acids in marine oils) or for squalene. Although the 0.1, 0.5, and 1.0% of added squalene produce visible peaks in Figure 1, the electronic integration gives a

signal for approximately 0.03% of pre-existing squalene converted to squalane in the GLC analysis of the totally hydrogenated esters of the starting oil (Fig. 2). Clearly 0.1, 0.5, and 1.0% squalane are visible in this figure in the position indicated by a squalane standard and just ahead of the 20:0 peak. As expected from previous experience with Adam's catalyst (14) hydrogenation of the methyl esters of seal oil fatty acids was complete, and the baseline was free of extraneous peaks such as might have come from incomplete hydrogenation of fatty acids or of squalene.

Table 1 gives the results of the analysis of the totally hydrogenated seal oil of Newfoundland origin. The area percentages of the squalane peaks are obviously exaggerated by about 25% over the weights of squalene added to the original oil. Of this, 17% is accounted for because of the theoretical FID response factors. Squalane gives a higher response than methyl esters because all carbon atoms yield ions (12,15,16). A further correction of about 5% arises from the removal of the glycerol from the oil (triacylglycerol) analysis as the computer program reports only weight percentages of fatty acids reaching the detector, leading to a total theoretical correction of 1.22. De Leonardis *et al.* (7) reported an experimental correction factor of 1.32 for squalene converted to squalane in analyses of unhydrogenated and hydrogenated methyl esters of fatty acids of olive oil. In their work on olive oil they employed a similar polyglycol capillary column for GLC. They also showed that squalene, as expected, gave a linear response in the FID.

The sample of seal oil from the Magdalen Islands showed

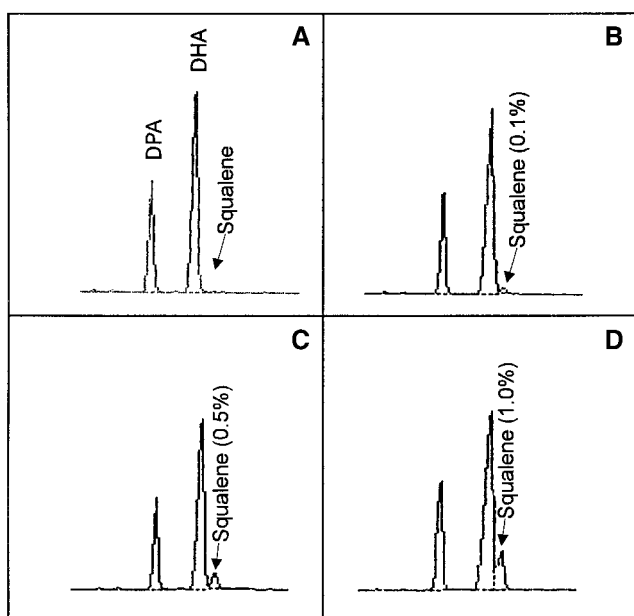


FIG. 1. Parts of gas-liquid chromatography (GLC) analyses of methyl esters of fatty acids of seal oil without added squalene and with 0.1, 0.5, and 1.0% squalene added. DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

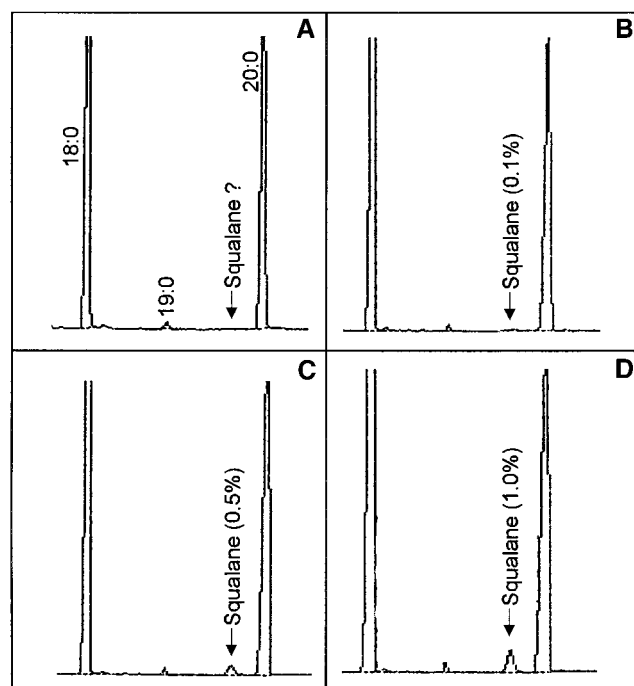


FIG. 2. Parts of GLC analyses of methyl esters of totally hydrogenated fatty acids of seal oil and of squalane, including that from squalene added at 0.1, 0.5, and 1.0%. For abbreviation see Figure 1.

TABLE 1
Composition (% w/w) of Hydrogenated Newfoundland Seal Oil Fatty Acids (1998 production) When Various Concentrations of Squalene Were Added to the Original Oil (mean – SD, n = 3)

Fatty acid ^a	Squalene added			
	0.0%	0.1%	0.5%	1.0%
14:0	5.40 ± 0.11	5.41 ± 0.02	5.35 ± 0.12	5.18 ± 0.16
115:0	0.25 ± 0.03	0.22 ± 0.06	0.22 ± 0.06	0.21 ± 0.05
Al15:0	0.21 ± 0.04	0.13 ± 0.05	0.14 ± 0.06	0.12 ± 0.04
15:0	0.47 ± 0.11	0.47 ± 0.11	0.46 ± 0.10	0.43 ± 0.10
116:0	0.09 ± 0.08	0.13 ± 0.04	0.13 ± 0.03	0.12 ± 0.03
16:0	25.24 ± 0.50	25.14 ± 0.46	24.78 ± 0.19	24.37 ± 0.28
7ME16:0	0.45 ± 0.05	0.48 ± 0.02	0.45 ± 0.03	0.45 ± 0.03
117:0	0.10 ± 0.07	0.13 ± 0.02	0.14 ± 0.03	0.13 ± 0.02
Al17:0	0.13 ± 0.09	0.12 ± 0.03	0.12 ± 0.03	0.11 ± 0.03
17:0	0.54 ± 0.06	0.56 ± 0.05	0.56 ± 0.05	0.55 ± 0.05
118:0	0.04 ± 0.08	0.10 ± 0.03	0.11 ± 0.03	0.10 ± 0.03
18:0	29.81 ± 0.56	29.61 ± 0.32	29.40 ± 0.25	29.21 ± 0.01
19:0	0.25 ± 0.01	0.25 ± 0.02	0.25 ± 0.02	0.24 ± 0.02
Squalane	0.03 ± 0.02	0.15 ± 0.02	0.65 ± 0.03	1.32 ± 0.03
20:0	20.62 ± 0.11	20.57 ± 0.05	20.59 ± 0.17	20.63 ± 0.35
21:0	0.52 ± 0.03	0.52 ± 0.01	0.52 ± 0.01	0.54 ± 0.03
22:0	15.57 ± 0.29	15.60 ± 0.34	15.73 ± 0.04	15.91 ± 0.34
24:0	0.39 ± 0.05	0.40 ± 0.03	0.40 ± 0.01	0.40 ± 0.01

^aAl, anteiso; I, iso; 7ME, 7-methyl.

0.30% squalene, corrected from the analysis of squalene, and the 1961 Nova Scotia sample showed 0.05%. The latter probably was produced from Newfoundland pelts. There is no way to differentiate the Arctic feeding area of the harp seals taken in the Gulf of St. Lawrence to the west of Newfoundland from those taken from east of Newfoundland at the harvesting area called the “front.” Grey seals, *Halichoerus grypus*, are likely to be temporary or permanent residents in the Gulf of St. Lawrence and are often harvested by hunters from the Magdalen Islands (17), but at a different time of the year. The 1961 seal oil produced in Nova Scotia from harp seal pelts had a fatty acid composition remarkably similar to that of the 1998 sample from Newfoundland, suggesting little change in dietary fats and fatty acids over nearly 30 yr. This included the contributions from the highly unsaturated fatty acids, so little or no oxidation had taken place.

In 1977 a Canadian Fisheries and Marine Services Technical Report (1) was published that described the hydrocarbons in marine organisms and sediments off West Greenland. This baseline survey was initiated by three Danish bodies (Greenland Fisheries Investigation, Charlottenlund; Water Quality Institute, Soborg; National Food Institute, Horsholm). Among the hydrocarbons identified and commonly reported in this survey of diverse organisms, both pristane (2,6,10,14-tetramethylpentadecane) and squalene were ubiquitous, but squalene was usually present at only a few ppm/g of tissue, except in the one case of cod livers (1). This area includes the summer and fall feeding grounds of the harp seal, and there is no obvious reason for squalene to accumulate in seal blubber from any particular exogenous source.

Squalene is a normal component of parts or organs of most mammalian bodies as it can be made in most cells and circulates freely in the blood stream (18–21). It is an integral part

of cholesterol biosynthesis (19,22). Human health benefits seem dubious, and absorption in the intestines is both incomplete and confusing (19,22). It was therefore surprising to learn that a Canadian encapsulated seal oil product was being promoted in Asia on the basis of the health benefits of the squalene content, especially if a considerable proportion of the low level originally present would be removed during refining by deodorization. Possibly the presumed benefits spill over from the increasing use of squalene in cosmetics (23,24), since squalene is biosynthesized in skin cells (25).

Squalene should be subject to oxidation, along with the polyunsaturated fatty acids of marine oils, as shown by the work of De Leonardis *et al.* (7) and of Psomiadou and Tsimidou (26) with olive oil. Possibly it may also be a useful sacrificial antioxidant in raw seal oil (10). Once hydrogenated, however, both the fatty acid methyl esters and the squalane are perfectly stable. A further advantage is that the mass spectrum of the fully saturated squalane could, if required, also be more satisfactory than that of squalene, which is subject to extreme fragmentation. The increments in the seal oil samples with added squalene respond as squalane as expected (Table 1). The simplicity of the GLC approach is especially suited to marine oils where the multiplicity of components makes it difficult to state that squalene in any GLC analysis of whole oil fatty acid methyl esters does not coincide with some minor fatty acid component. The GLC retention time of squalane can in any case be easily manipulated in GLC because of the chemical/physical differences of a saturated hydrocarbon from the saturated methyl esters. Therefore, this method of squalene analysis by hydrogenation has several additional advantages over the conventional “unsaponifiable” approach.

The total hydrogenation of the Carino 1998 Newfoundland

raw seal oil sample gave squalane at a remarkably lower level (~0.03%) than the squalene figure (0.59%) previously reported (10) for harp seal oil. The 1961 Nova Scotian sample (with 0.05%) agrees with this result. The Gulf of St. Lawrence and Magdalen Islands 1998 seal oils suggest, however, that some natural variation can be expected even for one species, depending mostly on the diet of the seals.

Olive oil. The proposed hydrogenation–GLC technology was satisfactory for a marine oil and has already been investigated for olive oil (7). However, many common vegetable oils contain squalene (8), including palm kernel and palm oil (27,28). Squalene has been reported in deodorizer condensates from refining canola and soybean oils (29). Normally, extra virgin or even virgin olive oils are not deodorized (30), but nonpremium grades are refined and the condensates have been examined for squalene by conventional methods (31,32). Artifacts of squalene can be produced during the refining and deodorization common with vegetable oils (32). Doubts have recently been cast on an antioxidant role for squalene in olive oil (26). However, it is interesting that the two common vegetable oils best known for squalene, olive and palm, are produced from drupes and not seeds. Both fruits have to be handled promptly because of *in situ* lipoxygenase activity (33). The presence of squalene thus suggests a function as an antioxidant. Rice bran oil is another vegetable oil containing considerable squalene (*ca.* 0.5%), probably for the same reason.

The popularity of olive oil as a presumed healthful dietary fat, despite low levels of both linoleic and α -linolenic acids, created the original concept of the Mediterranean diet rich in olive oil (30,34). However, the olive oil basis was subsequently modified with canola oil to include more linoleic and α -linolenic acids (35), and recently this Mediterranean diet has been reported successful in clinical trials (36). Miettinen and Vanhanen (19) have used “rapeseed” oil low in squalene as a source of monoethylenic fatty acids in evaluating the effect of squalene on blood lipids and found some changes with 1 g squalene/d, but serum sterols were “normal” with 0.5 g squalene/d. Their further research into squalene levels and coronary artery disease (20) suggests a correlation in postmenopausal women.

In the United States, sales of extra virgin olive oil have increased 51% since 1995. Imports of extra virgin oil have increased 12% annually since 1991 (37). For readers of the *Journal of the American Oil Chemists' Society* with interest in oil identification and/or quality control, and since an olive oil industry is growing in California, we consider it useful to illustrate the hydrogenation of olive oil for squalene content. Figure 3 shows that, under normal conditions for the analysis of fatty acids of olive oil by GLC, the squalene present was a small and late-eluting component, although the peak area was greater by a factor of eight compared to the immediately preceding 24:0 peak. Hydrogenation gave squalane in the position immediately preceding the 20:0 peak (Fig. 3, inset). The corrected content of squalene measured as squalane in the Greek olive oil purchased in Canada was 0.37% of total fatty

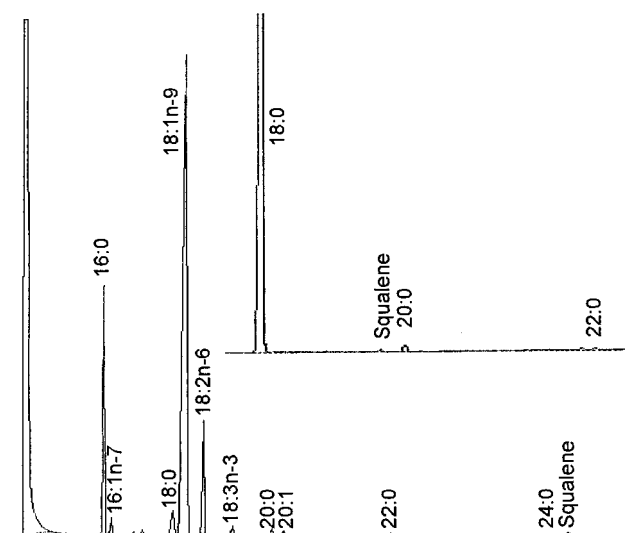


FIG. 3. GLC record of the methyl esters of extra virgin olive oil fatty acids showing (extreme right) the relatively small peaks for 24:0 and for squalene, and (inset) part of the GLC record of analysis of the hydrogenated methyl esters of fatty acids from the extra virgin olive oil to show the position and proportion of squalane relative to 20:0. For abbreviation see Figure 1.

acid area and that of the Cretan olive oil was 0.40%, well within the normal squalene content range for European olive oils. The unrefined retail palm oil examined had about 0.05% squalene, but palm oil is both fractionated and refined to produce a variety of products, so this amount is not necessarily typical of all raw oil. What is of interest is that the hydrogenated methyl esters of both olive oil and palm oil were free of known or unknown components such as iso or anteiso fatty acids in the region where squalane was found.

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