

# Rapid Quantitative Determination of Squalene in Shark Liver Oils by Raman and IR Spectroscopy

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**Abstract** Squalene is sourced predominantly from shark liver oils and to a lesser extent from plants such as olives. It is used for the production of surfactants, dyes, sunscreen, and cosmetics. The economic value of shark liver oil is directly related to the squalene content, which in turn is highly variable and species-dependent. Presented here is a validated gas chromatography-mass spectrometry analysis method for the quantitation of squalene in shark liver oils, with an accuracy of 99.0 %, precision of 0.23 % (standard deviation), and linearity of >0.999. The method has been used to measure the squalene concentration of 16 commercial shark liver oils. These reference squalene concentrations were related to infrared (IR) and Raman spectra of the same oils using partial least squares regression. The resultant models were suitable for the rapid quantitation of squalene in shark liver oils, with cross-validation  $r^2$  values of >0.98 and root mean square errors of validation of  $\leq 4.3$  % w/w. Independent test set validation of these models found mean absolute deviations of the 4.9 and 1.0 % w/w for the IR and Raman models, respectively. Both techniques were more accurate than results obtained by an industrial refractive index analysis method, which is used for rapid, cheap quantitation of squalene in shark liver oils. In particular, the Raman partial least squares regression was suited to quantitative squalene analysis. The intense and highly characteristic Raman bands of squalene

made quantitative analysis possible irrespective of the lipid matrix.

**Keywords** Squalene · Shark liver · Raman · Infrared · Partial least squares regression · Gas chromatography · Mass spectrometry

## Abbreviations

ATR	Attenuated total reflectance
CCD	Charge coupled device
DAGE	Diacylglycerol ether
GC	Gas chromatography
HPLC	High performance liquid chromatography
ICH	International Conference on Harmonization of Technical Requirements
IR	Infrared
MAD	Mean absolute deviation
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NIPALS	Non-iterative partial least squares
PLS-R	Partial least squares regression
PUFA	Polyunsaturated fatty acids
RMSEC	Root mean squared error of calibration
RMSEV	Root mean squared error of validation
SNV	Standard normal variate
TAG	Triacylglycerol

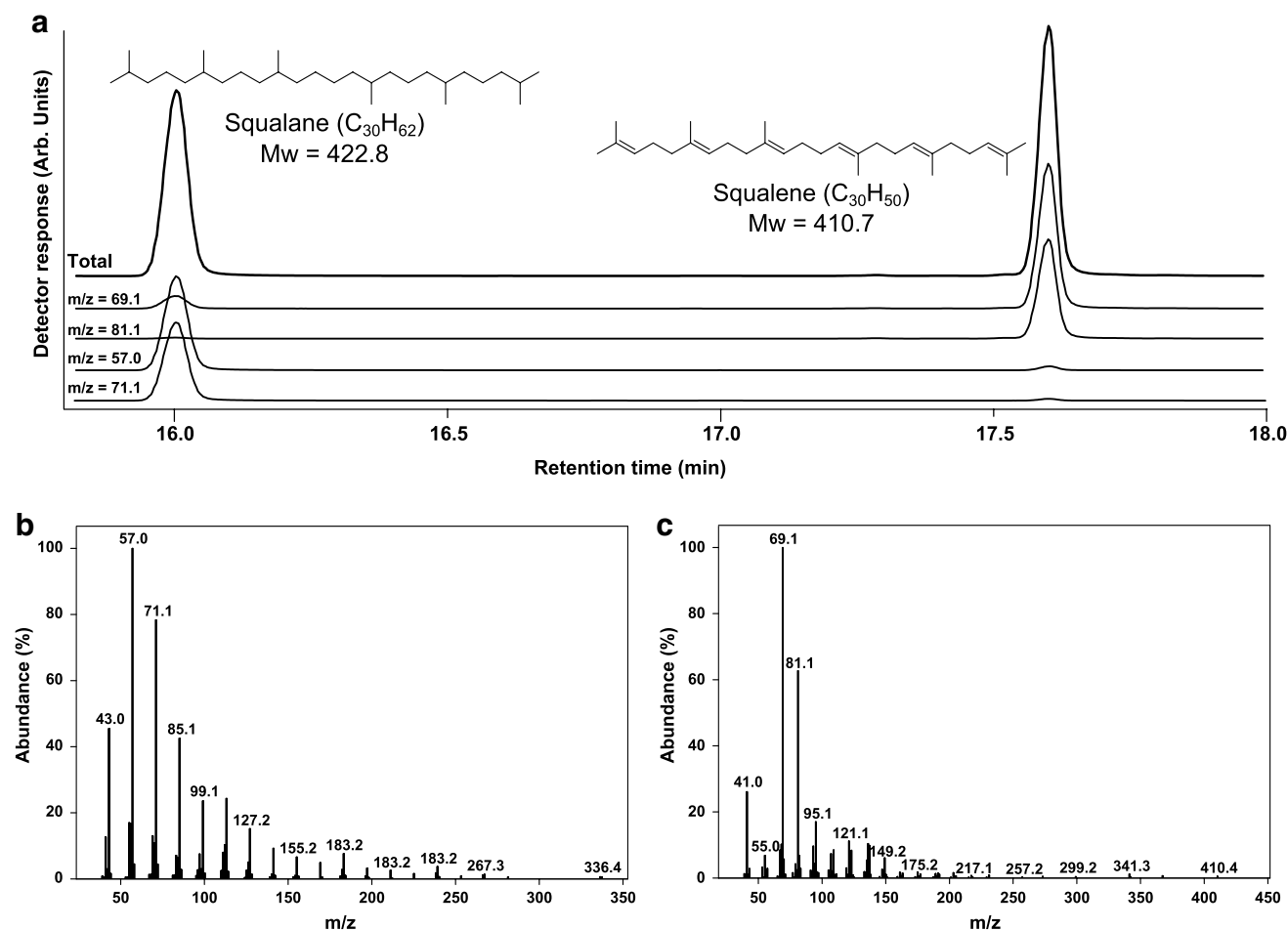
## Introduction

Squalene is a highly unsaturated, unconjugated triterpene (Fig. 1a), sourced primarily from shark liver oils and to a lesser extent from plant sources [1]. It is used in the production of surfactants, dyes, and sunscreens [2]. It has also been associated with a range of bioactivities and therapeutic uses

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**Fig. 1** Summary of gas chromatography–mass spectrometry (GC–MS) analysis method for quantitation of squalene in shark liver oil. **a** Retention time range showing the elution times of squalane (16.0 min) and squalene (17.7 min) with the selective ion monitor

(SIM) responses for  $m/z = 69.1, 81.1, 57.0$  and  $71.5$ , **b** mass spectrum of squalane with highlighted SIM and **c** mass spectrum of squalene with highlighted SIM

[3]. Squalene is the main raw material for the production of its fully saturated analogue squalane, an emollient used by the cosmetics industry (Fig. 1a) [4]. The squalene concentration of shark liver oil is highly species-dependent, varying from 0 to 90 % w/w [5–9]. The low density of squalene ( $0.858 \text{ g cm}^{-3}$ ) is believed to aid deep-sea sharks in maintaining neutral buoyancy [10]. As such, the livers of deep-sea species tend to have higher squalene concentrations than those of shark species living at shallower depths [5–9]. The other major components of shark liver oil are diacylglycerol ethers (DAGE) and triacylglycerols (TAG) [5–9]. DAGE, like squalene, is believed to function as a buoyancy aid (density =  $0.89 \text{ g cm}^{-3}$ ). The concentration of DAGE in shark liver oils tends to be negatively correlated to the concentrations of squalene in shark species ( $r^2 = 0.83$ ) [5].

The value of shark liver oil is directly related to its squalene composition, necessitating reliable analytical methodologies for its measurement [2]. A recent review

summarized squalene analysis methods, which is most commonly achieved by gas chromatography (GC) and high performance liquid chromatography (HPLC), with various detection methods [1]. While these chromatographic techniques produce accurate results, the cost of purchasing, operating, and maintaining GC or HPLC systems can outweigh their benefits. Another analytical method for squalene analysis is thin layer chromatography with flame ionization detection (commonly referred to as “Iatroscan” analysis) [5, 8]. However, this approach is not specific and cannot discriminate squalene from other hydrocarbons that may be present in the oil. Historically, quantitative spectrophotometry with detection at 400 nm has been used to estimate squalene concentrations [11]. That method required samples to be dried, treated with  $\text{H}_2\text{SO}_4$ , and developed [11]. A more recent methodology involved the use of elemental analysis with isotope-ratio mass spectrometry (MS) detection [12]. In industrial settings, the squalene content

**Table 1** Squalene composition of validation standards by gas chromatography-mass spectrometry (GC-MS)

Validation standards (% squalene)	GC-MS replicate analyses					Average % Recovery ( $n = 4$ )	Standard deviation ( $n = 4$ )
	Prep. 1 Inj. 1	Prep. 1 Inj. 2	Prep. 2 Inj. 1	Prep. 2 Inj. 2	Average ( $n = 4$ )		
9.8	9.62	9.64	9.57	9.56	9.6	97.9	0.04
30.3	30.38	30.25	30.66	30.55	30.5	100.5	0.18
49.8	49.8	50.01	49.92	49.64	49.8	100.1	0.16
70.1	69.8	69.69	69.16	69.22	69.5	99.1	0.32
89.9	88.14	87.91	87.085	87.68	87.7	97.6	0.45
					Average ( $n = 5$ )	99.0	0.23

of shark liver oils is sometimes calculated from the oil's refractive index [13]. While refractive index is a cheap and fast analytical method, it suffers from inaccuracy and temperature sensitivity. Furthermore, refractive index is a “black-box” methodology, which provides no additional corroborating evidence relating to oil composition.

The Raman and IR spectra of squalene have recently been investigated experimentally and assigned using density functional theory calculations [14]. While the IR spectrum contains no unusual or exceptionally characteristic bands, the Raman spectrum of squalene has an unusually intense band at  $1670\text{ cm}^{-1}$ , arising from the cumulative intensity of the symmetric stretching of the compound's six double bonds (Fig. 1a) [14]. A recent review of the Raman spectra of lipids showed that the C-H stretching region of these compounds ( $3100\text{--}2700\text{ cm}^{-1}$ ) is generally far more intense than the vibrational modes occurring below  $1800\text{ cm}^{-1}$  [15]. As such, the squalene Raman band at  $1670\text{ cm}^{-1}$  is unusually intense and, because the double bonds are tri-substituted, occurs at a higher energy than double bonds found in MUFA and PUFA [15]. This potentially makes the band analytically useful. Indeed, recent reports that use Raman spectroscopy for the analysis of olive oils have observed this characteristic squalene band, despite the compound being present at less than 1 % in these oils [16–18]. Therefore, Raman spectroscopy may be suitable for the selective analysis of squalene in lipid matrixes. It is noted that the squalene band at  $1670\text{ cm}^{-1}$  is much less intense than the vibrational bands associated with highly conjugated terpenes derivatives such as  $\beta$ -carotene [19, 20].

This report describes a validated GC-MS method for accurate and precise quantitation of squalene in shark liver oils. The method was based on a previously published report by Lanzon et al. [21]. This method was used to measure the squalene content of 16 commercial shark liver oils. The Raman and IR spectra of the same shark liver oils were related to the GC-MS reference results using partial least squares regression (PLS-R). The quantitative performance of the resultant spectroscopic models was appraised by both cross-validation and test-set validation. Results from the spectroscopic analyses were compared with results generated by an industrial refractive index method [13]. These

spectroscopic models were developed as a compromise between the rapid, but unreliable, refractive index analysis method and the accurate, but more expensive, gas chromatography analysis method.

## Materials and Methods

### Chemicals and Standards

Squalene ( $\geq 98\%$ ) and squalane (99 %) were sourced from Sigma-Aldrich. GC grade hexane and methanol (Merck), and MilliQ™ (Millipore) grade water were used. Analytical reagent grade ethanol (96 %), KOH, and  $\text{Na}_2\text{SO}_4$  were sourced from Merck.

### Samples

Sixteen shark liver oils, chosen to represent a wide range of squalene concentrations and diverse oil compositions, were provided for this study by SeaDragon Marine Oils Ltd® (New Zealand). Oils were sourced from a variety of shark species from different global locations. Oils were either unmodified or had undergone various chemical processing, e.g. steam stripping, molecular distillation, and bleaching. The processing methods commonly applied to shark liver oils have recently been reviewed [1]. A series of validation standards were prepared by adding accurately weighed amounts of squalene to school shark (*Galeorhinus galeus* L.) oil (Table 1). The school shark oil itself contained only trace amounts of squalene ( $<0.1\%$ ). An additional “check” sample was prepared from squalene and school shark oil (50.2 % w/w squalene) and analysed periodically throughout GC-MS analysis runs.

### Gas Chromatography-Mass Spectrometry: Sample Preparation

An internal standard solution was prepared by dissolving squalane in hexane ( $100\text{ mg mL}^{-1}$ ). Shark liver oil (100–200 mg) was accurately weighed into a 15-mL plastic

centrifuge tube and dissolved in 4 mL of hexane and 1 mL of ISS. One milliliter of a 2 M KOH solution in methanol was added to the tube. The tube was capped and the contents mixed for 90 s (vortex mixer) at room temperature to effect methanolysis of acylglycerides in the oils. After standing for 10 min, the reaction mixture was centrifuged to achieve clean phase separation, and the lower methanolic layer was removed and discarded. The remaining hexane layer was washed twice with 1:1 ethanol–water (4 mL), with the lower aqueous layer being removed and discarded between washes. The washed hexane extract was then removed, dried with anhydrous  $\text{Na}_2\text{SO}_4$  (c. 500 mg), and diluted 1000 $\times$  for analysis by GC-MS. This method was based on a previously published report [21]. Calibration standards were prepared by substituting accurately measured quantities of squalene standard (100 mg  $\text{mL}^{-1}$  in hexane) for shark liver oil samples. The final squalene concentrations in the calibration standards were 0, 8, 16, 24, 32, and 40 ppm.

### Gas Chromatography-Mass Spectrometry

GC-MS analysis was performed using a Shimadzu QP-2010 instrument equipped with a Restek Rxi<sup>®</sup>-5Sil MS, 30 m  $\times$  0.25 mm ID column (5 % diphenyl/95 % dimethylpolysiloxane). Injections (1  $\mu\text{L}$ , splitless, 300  $^\circ\text{C}$ , sampling time 2 min) were performed using a PAL auto-sampler. The GC oven temperature was held at 60  $^\circ\text{C}$  for 2.5 min, ramped from 60 to 240  $^\circ\text{C}$  (20  $^\circ\text{C min}^{-1}$ ), then from 240 to 280  $^\circ\text{C}$  (5  $^\circ\text{C min}^{-1}$ ), and finally to 300 (20  $^\circ\text{C min}^{-1}$ ), where it was held for 2 min. The carrier gas was helium, with a column flow rate of 2  $\text{mL min}^{-1}$  maintained using linear velocity control (total flow 37  $\text{mL min}^{-1}$ ). Detection was facilitated by electron impact mass spectrometry at 70 eV (ion source 230  $^\circ\text{C}$ , transfer line 270  $^\circ\text{C}$ ). Selective ion monitoring was performed at  $m/z = 57.0, 69.1, 71.1, \text{ and } 81.1$  (Fig. 1).

Validation was performed in agreement with the general guidelines supplied by The International Conference on Harmonization of Technical Requirements (ICH) [22]. Squalene recovery (extractability) was determined by analysing the validation standards and comparing the results with their known squalene concentrations, listed in Table 1. This analysis was also used to determine analytical accuracy and precision over a range of squalene concentrations. Intermediate precision was determined by preparing and analysing shark liver oils in triplicate on three separate days by two different analysts. Throughout these analyses, the same “check” sample was periodically analysed to demonstrate sample stability and method reproducibility.

### IR Analysis

IR absorption spectra were acquired in the region of 4000–400  $\text{cm}^{-1}$  using a Bruker ALPHA Fourier transform IR

spectrometer. Oils were presented directly onto the attenuated total reflectance (ATR) diamond accessory and 16 co-added scans were acquired with a spectral resolution of 4  $\text{cm}^{-1}$ . Spectra consisted of 2514 data points. Three spectral replicates were acquired for each oil sample and spectral backgrounds were acquired at regular 5-min intervals.

### Raman Analysis

Raman spectra were acquired using a Senterra Raman microscope equipped with an Olympus BX microscope with  $\times 20$  objective lens. Raman scattering was generated using a 785 nm diode laser at 100 mW. Raman spectra were measured as Stokes-shifted radiation from the laser line in the range of 3200–90  $\text{cm}^{-1}$  with a spectral resolution of 9–18  $\text{cm}^{-1}$ , using OPUS 6.5 software. Detection was facilitated by dispersing Raman-shifted radiation onto a CCD detector using a grating (1200 grooves  $\text{mm}^{-1}$ ). Shark liver oils (approx. 50  $\mu\text{L}$ ) were presented in aluminium divots and analysed in triplicate. Spectra were the average of 100  $\times$  2 s co-additions and consisted of 6221 data points.

### Spectral Preprocessing and Multivariate Analysis

Preprocessing and multivariate analysis were performed using the Unscrambler<sup>®</sup> v10.3 software. IR spectra underwent a standard normal variate (SNV) transformation to compensate for inter-sample absorbance intensity and y-scattering effects. These spectra were subjected to a 15-point, second order gap derivative to remove baseline features, and the spectral ranges from 3050 to 2670 and 1800 to 500  $\text{cm}^{-1}$  were used to generate the PLS-R model (908 data points).

Raman spectra were subjected to a SNV transformation to normalize inter-sample spectral intensities and a 25-point, second order gap derivative to remove baseline features. The combined spectral ranges from 3050 to 2700 and 1800 to 400  $\text{cm}^{-1}$  were used to generate PLS-R models (3503 data points).

PLS regression models were generated using the non-iterative partial least squares (NIPALS) algorithm. Full, “leave-one-out” cross-validation was performed on each model. The IR and Raman models were subsequently used to predict squalene concentrations in the validation samples. Analysis of these samples constituted an independent test set validation of the spectroscopic models.

### Refractive Index Analysis

The refractive indices of the shark liver oil sample set and validation standards were measured using an Atago<sup>®</sup> PAL-RI “Pocket” refractometer. The average squalene content ( $n = 3$ ) was determined using the method previously described by Batista and Nunes [13].

## Results and Discussion

### GC-MS Results and Method Validation

To account for losses throughout the multi-step sample preparation, a fixed quantity of the squalane ISS was added to each shark liver oil sample. Squalane was chosen because it possesses similar physical properties to squalene (i.e. high boiling point, low polarity, and similar molecular mass), was chromatographically resolved (Fig. 1a), and was commercially available. To enhance analytical sensitivity and selectivity MS detection was performed using selected ion monitoring. Four ion channels were monitored: the two most abundant ions in the squalene mass spectrum at 69.1 and 81.1 (Fig. 1b), and the two most abundant ions in the mass spectrum of squalane at 57.0 and 71.1 (Fig. 1c). This approach provided excellent selectivity and allowed detection of squalene at concentrations of approximately 0.4 ppm. The ratio of the squalane peak area to squalene peak is defined here as the squalene response, which was related to the squalene concentration using a six-point calibration curve. Squalene response was linear ( $r^2 > 0.999$ ) from 0 to 40 ppm and the best-fit line passed easily through the origin. Taking into account the preparative dilutions, this range equated to squalene concentrations of 0–100 % w/w in undiluted shark liver oils.

To assess the recovery, accuracy, and precision of the GC-MS analysis method, duplicate preparation and analysis of the five validation standards was performed. The average recovery for these samples was 99.0 % (Table 1). These results also demonstrated the accuracy of the method, i.e. the closeness of measured values to the known concentrations. Precision was assessed by measuring the deviation of results from duplicate injections of duplicate preparations of the five validation samples. The average standard deviation of the replicate analyses ( $n = 4$ ) of the five validation samples was 0.23 % w/w (Table 1). Reproducibility was demonstrated by replicate analysis of a check sample, which was periodically analysed throughout the GC-MS analyses. The squalene concentration of this sample was 50.2 % w/w. The average result of the GC-MS analysis of this sample was  $50.16 \pm 0.25$  % w/w ( $n = 13$ ).

Intermediate precision was measured by comparing results for the squalene composition of the shark liver oil sample set by “analyst 1 on day 1” with results from independent preparations of the same samples by “analyst 2 on day 2”. The inter-analyst/inter-day analyses were in good agreement (103.4 %,  $n = 16$ ) with an average standard deviation of 0.57 % w/w (Table 2). The average squalene content ( $n = 3$ ) of these analyses were used as reference data for the spectroscopic PLS-R models described below (Table 2).

**Table 2** Squalene composition of shark liver oil samples by gas chromatography-mass spectrometry (GC-MS)

Sample	Analyst 1 Day 1 <sup>a</sup>	Analyst 1 Day 2 <sup>a</sup>	Analyst 2 Day 3 <sup>a</sup>	Average ( $n = 6$ )	Intermediate precision <sup>b</sup>	SD ( $n = 6$ )
S01	88.2	87.8	85.6	87.2	100.9	1.28
S02	37.9	37.4	38	37.8	99.7	0.46
S03	29.8	29.8	30	29.9	99.8	0.13
S04	77.6	77.8	76.7	77.4	100.4	0.52
S05	0.5	0.5	0.7	0.6	87.7	0.13
S06	6.8	6.9	7.4	7.1	97.5	0.27
S07	54.4	54.3	54.6	54.4	99.8	0.20
S08	3.3	3.3	3.5	3.4	97.9	0.13
S09	8.5	8.7	8.7	8.6	99.5	0.13
S10	97.2	94.6	94.2	95.3	100.6	1.48
S11	97.2	96.7	94.3	96.1	100.9	1.49
S12	83.7	84.2	81.7	83.2	100.9	1.21
S13	83.2	83.1	82.6	82.9	100.2	0.33
S14	67.2	67.2	67.1	67.2	100.0	0.12
S15	17.1	17.0	17.2	17.1	99.7	0.13
S16	6.9	6.8	7.2	7.0	98.3	0.18
				Average ( $n = 16$ )	99.0	0.51

SD standard deviation

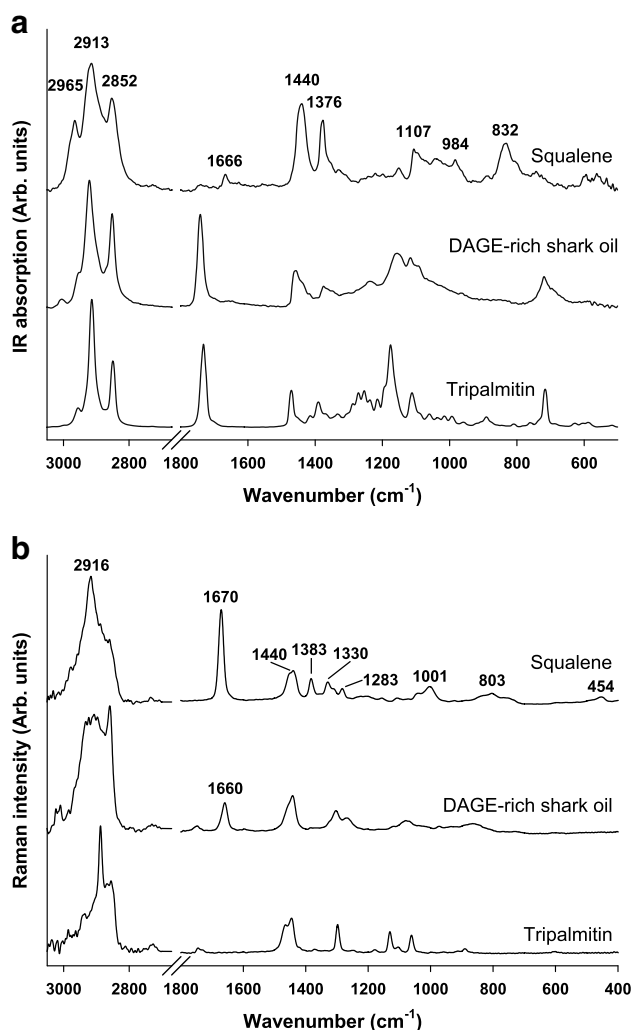
<sup>a</sup> Average result of two injections

<sup>b</sup> Analyst 2 result as a % of the average analyst 1's results ( $n = 2$ )



## IR and Raman Spectra of Shark Oils

The IR spectrum of squalene had three intense C–H stretching bands at 2965, 2913, and 2852  $\text{cm}^{-1}$  and four intense skeletal vibrational modes at 1440, 1376, 1107, and 832  $\text{cm}^{-1}$  (Fig. 2a). The low intensity band at 1666  $\text{cm}^{-1}$  due to C=C stretching was also of analytical importance. The IR spectra of a TAG and a shark oil rich in DAGE, which are the other main constituents of shark liver oils, are also shown in Fig. 2a. The IR spectra of these glycerol derivatives share many features, including the intense C–H stretching bands from 3000 to 2800  $\text{cm}^{-1}$ , the intense carbonyl stretches ( $\approx 1740$   $\text{cm}^{-1}$ ) and strong skeletal and hydroxyl bending modes at  $\approx 1468$ , 1165, 1112, and 720  $\text{cm}^{-1}$ . The full assignments of the IR spectra of these



**Fig. 2** **a** Infrared (IR) and **b** Raman spectra of squalene, triacylglyceride (TAG) and shark oil rich in diacylglyceride ether (DAGE). In the Raman spectra, the intensity of the region from 1800–400  $\text{cm}^{-1}$  is scaled  $\times 3$  relative to the region from 3050–2700  $\text{cm}^{-1}$

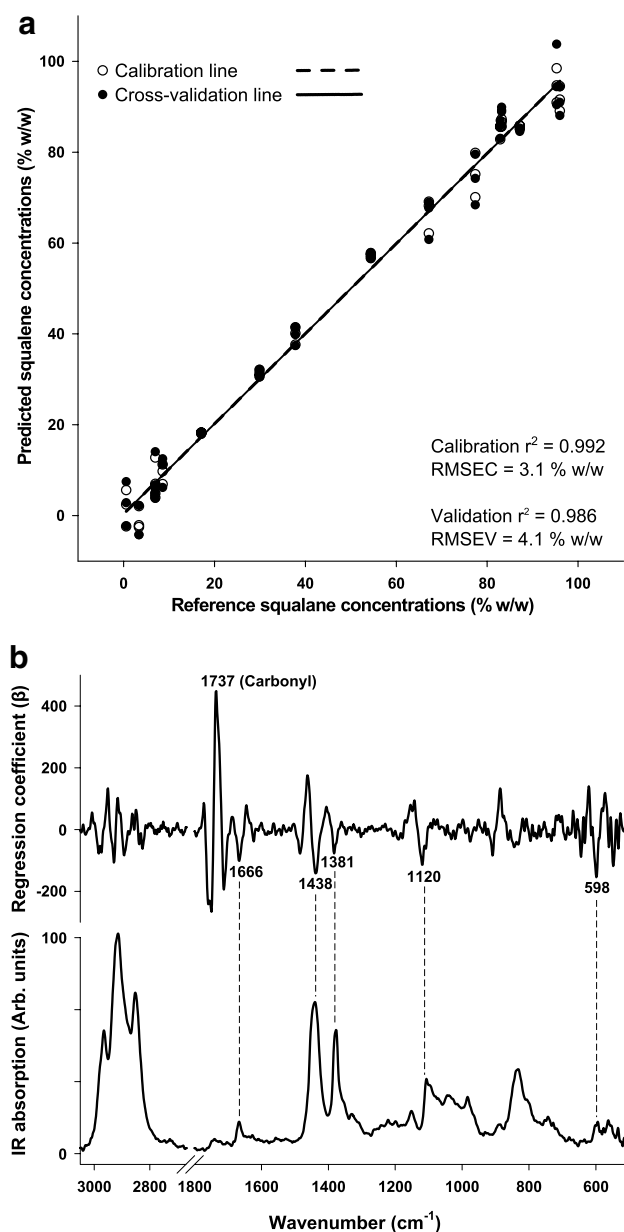
and related compounds are comprehensively described elsewhere [14, 23].

As is the case with most lipids, the Raman spectrum of squalene was dominated by the C–H stretching region (3000–2700  $\text{cm}^{-1}$ ) [15]. Therefore, for the purposes of presentation, the intensity of the spectral region from 1800 to 400  $\text{cm}^{-1}$  has been magnified  $3\times$  compared to the C–H stretching region (Fig. 2b). Some less intense, but analytically important, bands in the Raman spectrum of squalene occurred at 1440, 1383, 1330, 1283, 1001, 803, and 454  $\text{cm}^{-1}$  due to various skeletal stretching and bending modes. However, the most distinctive Raman band in the spectrum of squalene was observed at 1670  $\text{cm}^{-1}$  due to the additive intensity of the symmetric stretching of the six highly polarisable double bonds in the compound (Fig. 1a) [14]. These tri-substituted double bonds gave rise to a Raman band at slightly higher energy than those of di-substituted fatty acid double bonds [15]. As such, the squalene Raman band at 1670  $\text{cm}^{-1}$  could be used to distinguish squalene double bond stretches from normal fatty acid double bond stretching  $\approx 1640$ – $1660$   $\text{cm}^{-1}$  [15]. While the intensity of this band was high relative to those of other lipids, it was far less intense than double bond stretching signals associated with conjugated hydrocarbons such as  $\beta$ -carotene [15, 19].

The IR and Raman spectra of the other most common shark liver oil components (DAGE and TAG) are shown in Fig. 2b [5]. Besides the C–H stretching region, the most intense Raman bands in the DAGE-rich shark oil were observed at 1660, 1442, 1305, and 1266  $\text{cm}^{-1}$ , whereas the most intense TAG bands were found at 1446, 1298, 1130, and 1061  $\text{cm}^{-1}$  (Fig. 2b). Weak carbonyl stretching vibrations were observed for both TAG and DAGE samples at  $\approx 1750$   $\text{cm}^{-1}$ . The Raman spectrum of these and related compounds have recently been reviewed and assigned in detail [15]. The double bond stretching mode of the DAGE-rich shark oil is at 1660  $\text{cm}^{-1}$ . This illustrates the aforementioned distinction between di- and tri-substituted double bond stretching vibrational frequencies (Fig. 2b).

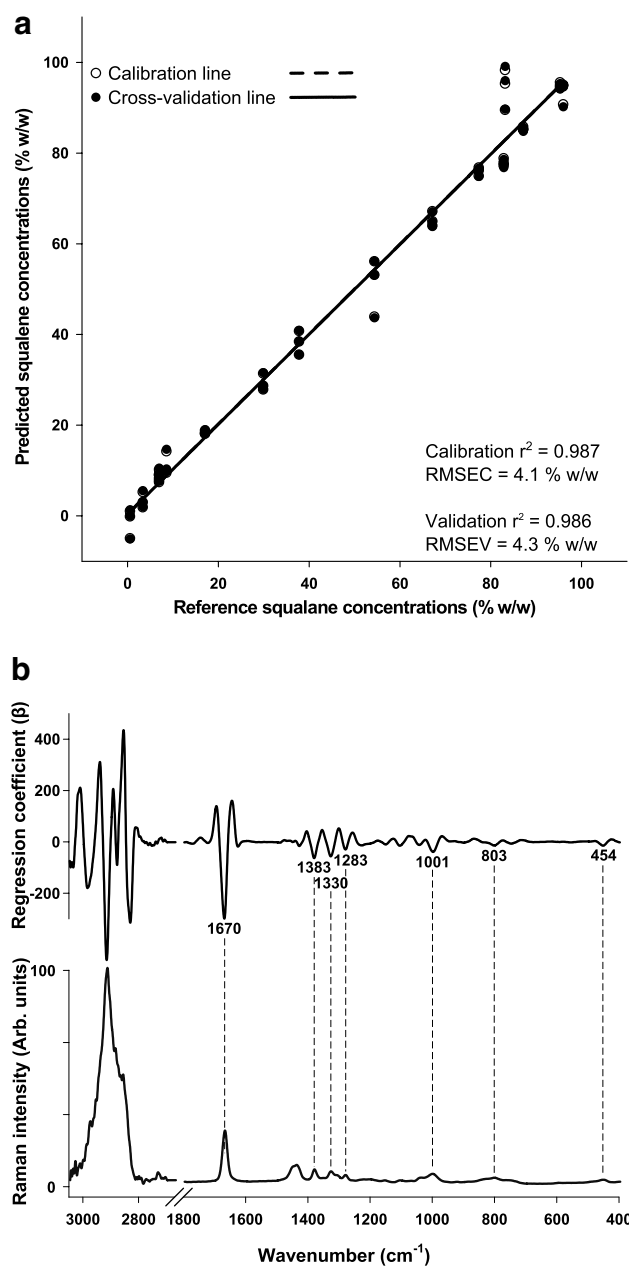
## Spectroscopic PLS-R Models

The optimal IR PLS-R model was produced by relating the GC-MS reference data (Table 2) to the IR spectral ranges from 3050 to 2670 and 1800 to 500  $\text{cm}^{-1}$  of the shark liver oils. The modelled relationship between the squalene reference concentrations by GC-MS and the predicted squalene concentrations by IR is summarized in Fig. 3a. The full, “leave-one-out” cross-validation of this model had an  $r^2 = 0.986$  and a root-mean-square error of validation (RMSEC) of 4.1 % w/w using three regression factors (Fig. 3a). The spectral variability responsible for the PLS-R model is summarized by the PLS regression coefficient ( $\beta$ ), which is related to the spectrum of squalene in



**Fig. 3** Summary of infrared (IR) partial least squares regression (PLS-R) model. **a** Calibration and cross-validation regression lines with model performance metrics inset and **b** the PLS-R model regression coefficient related to the IR spectrum of squalene from shark liver oils

**Fig. 3b.** It is evident that intensity variance of the squalene IR bands at 1666, 1440, 1376, 1107, and 832  $\text{cm}^{-1}$  influences the PLS-R model. However, the most notable feature in the IR PLS-R model regression factor is due to the carbonyl stretching of the glycerol derivatives at 1750  $\text{cm}^{-1}$  (Fig. 3b). This band is inversely loaded to the squalene bands, which means that, in addition to squalene vibrational bands, the IR model is using the “absence of glycerol derivatives” to estimate the squalene concentration.



**Fig. 4** Summary of Raman partial least squares regression model. **a** Calibration and cross-validation regression lines with model performance metrics inset and **b** the model regression coefficient related to the Raman spectrum of squalene from shark liver oils

The optimal Raman PLS-R model was produced from the spectral ranges from 3050 to 2700 and 1800 to 400  $\text{cm}^{-1}$ . The model had a cross-validation  $r^2 = 0.986$ , with a RMSEV of 4.3 % w/w (Fig. 4a). The model consisted of just a single PLS-R factor, which was strongly influenced by intensity variances at 2913, 1670, 1383, 1330, 1283, 1001, 803, and 454  $\text{cm}^{-1}$ —all of which are associated with the Raman spectrum of squalene (Fig. 4b). The strong influence of the symmetric double bond

**Table 3** Squalene content of validation standards by infrared (IR) and Raman partial least squares regression (PLS-R) models and refractive index

Validation standards Squalene (% w/w)	GC-MS results Squalene (% w/w)	IR		Raman		Refractive index	
		Squalene (% w/w)	Error (% w/w)	Squalene (% w/w)	Error (% w/w)	Squalene (% w/w)	Error (% w/w)
0	0	-1.3	0	-0.1	0.0	47.6	47.6
9.8	9.6	10.8	1.2	9.4	-0.2	48.7	48.7
30.3	30.5	34.3	3.8	33.3	2.8	64.9	55.3
49.8	49.8	55	5.2	51.3	1.5	78.5	48.0
70.1	69.5	77.9	8.4	69.2	-0.3	83.7	33.9
89.9	87.7	98.6	10.9	86.8	-0.9	93.2	23.7
Mean absolute deviation (MAD)			4.9		1.0		41.9

stretching band at  $1670\text{ cm}^{-1}$  was apparent (Fig. 4b). The IR and Raman regression factors ( $\beta$ ) shown in Figs. 3b and 4b, describe the explained spectral variance of the IR and Raman PLS-R models, respectively. A visual inspection of these figures suggests that the regression factor for the Raman model contained more squalene-specific explained variance than the regression factor for the IR model.

The validation samples were analysed using both spectroscopic PLS-R models and the refractive index method. These samples were diluted in school shark oil, which contained only trace amounts of squalene and had an oil composition distinct from the shark liver oil sample set. This provided a means of testing the selectivity of the squalene analysis methods. The squalene concentration of these samples by refractive index was highly inaccurate, with a mean absolute deviation (MAD) of 27.8 % w/w (Table 3). This was in line with refractive index results for the shark liver oils sample set, which produced values in the range of -45 to 112 % w/w (data not shown). While it has been shown previously that the squalene composition of shark liver oil can be correlated to refractive index [13], we found that the squalene content of processed shark liver oil could not be accurately measured using this approach. This was in line with results reported by industrial sources, who found that refractive index was highly unreliable for predicting the squalene content of processed shark liver oils, oils with unusual compositions and oils that have had been artificially enriched with squalene (personal communication). As such, refractive index analysis of squalene should be restricted to pure shark oils that have undergone minimal or no chemical processing.

The squalene composition of the validation samples were also measured using the IR and Raman PLS-R models. The results of these analyses represent an independent, test-set validation of the spectroscopic models (Table 3). The MAD of squalene concentrations in the validation samples was 4.9 % w/w using the IR model. This was a large improvement over the refractive index method for these samples. However, results from the IR model systematically over-estimated squalene content (Table 3). This was

probably due to interfering components in the school shark oil. As mentioned, the IR spectrum of squalene does not contain any characteristically intense or unusual vibrational bands, rendering it prone to interference from other lipid components with similar IR absorption bands. Analysis of the shark liver oil samples using the Raman PLS-R model produced more accurate results than results produced by the IR model. The MAD of squalene concentrations in the validation samples was 1.0 % w/w using the Raman model (Table 3). Unlike analysis using the refractive index method and the IR PLS-R model, the school shark oil matrix did not interfere with the accuracy of squalene quantitation by the Raman PLS-R model. As discussed above, this was probably due to the unusually strong and characteristic Raman spectral features of squalene, in particular the band at  $1670\text{ cm}^{-1}$ . As such, the Raman PLS-R model for squalene demonstrated better selectivity than both the refractive index and IR analysis methods. Raman spectroscopy is, therefore, better suited for the quantitative analysis of squalene than IR spectroscopy, especially in variable lipid matrixes. However, both spectroscopic techniques produced more accurate results than the refractive index methodology.

## Conclusions

A validated, quantitative GC-MS analysis method for squalene in pure and processed shark liver oils has been described. It has been demonstrated that quantitative analysis of squalene in shark oils can be performed rapidly using both IR and Raman spectroscopy in conjunction with PLS-R. These methods provide a compromise between the inexpensive, but unreliable, refractive index method and the highly accurate, but more expensive, chromatographic analysis methodologies. In particular, Raman spectroscopy was well suited to the analysis of squalene in shark liver oils. The strong performance of the Raman model is probably influenced by the relatively intense alkene stretching band of squalene, which helps to distinguish it from the



Raman spectra of most other lipids, including DAGE and TAG [15]. Based on our results, refractive index analysis is inappropriate for quantitation of squalene in variable oil matrixes, including processed shark liver oil samples.

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#### Compliance with Ethical Standards

**Conflict of interest** The authors declare no conflict of interest.

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