An improved RP-HPLC method for simultaneous analyses of squalene and cholesterol especially in aquatic foods

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Abstract A simple, rapid and simultaneous method for the quantitative evaluation of squalene and cholesterol in oil recovered from different body parts of fresh water and marine fish species was developed using reverse phase High performance liquid chromatography (RP-HPLC). A modified fractional crystallization method was developed using absolute ethanol as a solvent to obtain a fraction of squalene and cholesterol in the oil extracted from different body parts of fish samples. Elution of squalene and cholesterol was carried out isocratically with 100 % acetonitrile at 195 nm by diode array detector. Excellent linearity of the calibration curve was observed. The limit of detection for squalene and cholesterol were found to be 5.0 ng ml^{-1} and 1.0 µg ml^{-1} respectively. A possible correlation was established by determining the

ratio between cholesterol and squalene which was found to be ranging from 2 to 10. This method was successfully employed for the determination of squalene and cholesterol in the above mentioned samples.

Keywords Squalene · Cholesterol · RP-HPLC · Fractional crystallization

Introduction

Squalene is a natural lipid belonging to the triterpene hydrocarbon group (2,6,10,15,19,23 hexamethyltetracos-2,6,10,14,18,22-hexaene). It is a symmetrical 30-carbon compound having a polyprenyl chemical structure. It is pale yellow colored oil with faint odor and taste (Bhattacharjee and Singhal 2003). It has low density and is lighter than water with a specific gravity of 0.9. Squalene is known as a biochemical intermediate in the synthesis of cholesterol and other steroids. It is synthesized in liver and skin, further then transported in the blood (Reddy and Couvreur 2009). Sixty percent of dietary squalene is absorbed and distributed in human tissue (Vazquez et al. 2007). Squalene is used in clinical and daily usage as detoxification factor, skin and eye antioxidant, provide cells with oxygen as it has the ability to carry oxygen independent of hemoglobin, bactericidal and fungicidal agent, antistatic and emollient in pharmaceutical and cosmetics, fine chemical, magnetic tape and also as low temperature lubricants (Bhattacharjee and Singhal 2003). The name squalene was given because of its occurrence in shark liver oil (*Squalus* spp.), which is the richest and primary source of squalene (Vazquez et al. 2007). However, the limitation of squalene usage is because of the uncertainty of its availability as a result of international concern to protect marine animals (Bondioli

Highlights: • Simultaneous quantitative evaluation of squalene and cholesterol

- Improved sample preparation by modified fractional crystallization method
- Determination of squalene and cholesterol ratio
- RP-HPLC – Squalene elution at 14.2nd min and cholesterol at 11th min with sharp peaks and less interference.

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et al. 1993). Squalene can be found in substantial amounts in distillate residues of olive oil, soya bean oil, rice bran oil (RB DOD) & amaranth seed oil which have become attractive alternatives for shark liver oil. Isolation of squalene from micro-organisms is still under investigation and work is being carried out only at scientific levels.

Analysis of squalene has been achieved by various chemical and physical treatments such as Titrimetric, colorimetric, Molecular distillation (Choo et al. 2002), supercritical fluid extraction (Mendes et al. 2005), supercritical fluid chromatography (Guclu-Ustundag and Temelli 2005), crystallization (Lin and Koseoglu 2003), and chromatographic methods like high speed counter current chromatography (Lu et al. 2003), high resolution gas chromatography (Frega et al. 2003) and capillary column gas chromatography (de Blas and del Valle 1996). In all reported methods, tedious multi step sample preparation procedures seem to be inevitable. For example colorimetric method requires tedious drying since even traces of solvent interfere with color development. Sample preparation according to the AOAC method (AOAC 1999) involves multistep sample preparations like saponification, extraction of unsaponifiable matter using solvents, fractionating through column chromatography and other treatments. The same applies for both GC and LC methods. GC analysis of Squalene requires triacylglycerol removal, followed by fractionation of the unsaponifiable matter into several classes of compounds through column chromatography or TLC and in some cases derivatization. In the existing HPLC methods, treatments like alkaline digestion and distillation have been employed for both normal and reverse phase (RP). By using LC coupled with GC, direct analysis of squalene can be accomplished, if the capital cost, technical details and long elution time are not taken into account.

The aim of our study is to develop a rapid and simple method for simultaneous determination of squalene and cholesterol in different body parts of commercial fresh water and marine fish species. Fractional crystallization replaces alkaline treatment thus avoiding the use of larger quantities of toxic solvents and other tedious multistep processing techniques. This method was successfully applied for the determination of squalene in different body parts of commercial fresh water and marine fish species and other rich sources of squalene like olive oil, amaranth seed oil and RB DOD as well.

Materials and methods

Squalene (98 % pure) was purchased from Sigma-Aldrich (St Louis MO, USA); cholesterol 99 % pure was purchased from Sisco Research Laboratories, India. HPLC grade acetonitrile, methanol and ethanol were purchased from Merck, India. Analytical grade chloroform, methanol and sodium sulphate were purchased from Sisco Research Laboratories, India.

Samples used for analysis: Raw fish samples were procured from local market, transported and stored in cold condition (-20°C) until processing. Ten different fish species were randomly selected of which five were fresh water fish species: Rohu (*Labeo rohita*), Catla (*Catla catla*), Commom Carp (*Cyprinus carpio*), Tilapia (*Oreochromis mossambicus*), Mrigal (*Cirrhinus mrigala*). The other five were marine fish species: Pink Perch (*Nimepterus japonicus*), Sardine (*Sardinella longiceps*), Tuna (*Euthynus affinis*), Pangasius (*Pangasius pangasius*), and Mackerel (*Rastrelliger kanagurta*). Apart from these sources, other rich sources of squalene used for analysis were olive oil, RB DOD and oil from Amaranthus seeds which were procures from local supermarket. All the analyses were performed in triplicates.

Sample preparation

Extraction of oil from fish samples was carried out by separating different body parts of individual fish (head, meat, viscera and fins) and homogenizing separately in a mixer blender. Extraction procedure of oil was followed as described by Bligh and Dyer (1959). Homogenized samples were extracted in a solvent mixture of chloroform: methanol (2:1) overnight. The extract was then filtered through whatman filter paper 42 and residue was extracted twice in fresh solvent and then filtered. The filtrate was then dried over anhydrous sodium sulphate and concentrated in rotary flash evaporator (Hansvap, singapore). However the method suitability was tested by determining squalene content in samples other than aquatic sources like amaranth seed oil, Olive oil and RB DOD.

Preparation of standard

Stock solution of squalene and cholesterol standards were prepared in ethanol, later diluted in acetonitrile (mobile phase) to give final concentrations ranging from 1 to $150\text{ }\mu\text{g ml}^{-1}$ for squalene and 10 to $900\text{ }\mu\text{g ml}^{-1}$ for cholesterol.

Fractional crystallization

Fractionation of crude squalene was carried out by dissolving $500\text{ }\mu\text{l}$ of the extracted oil in 2 ml of absolute ethanol. After which the mixture was vortexed with the help of a mechanical shaker (vortexer) for 1 min to achieve proper homogenization and was kept for gravity separation till 6 h at ambient temperature. After separation, supernatant was collected and analyzed using RP-HPLC (Fig. 1). $20\text{ }\mu\text{l}$ of the ethanolic extract was dissolved in $500\text{ }\mu\text{l}$ of mobile phase (acetonitrile 100 %) and subjected to analysis. This separation method was employed for all the samples taken for study. Similarly a sample blank was prepared by dissolving $20\text{ }\mu\text{l}$ of ethanol in $500\text{ }\mu\text{l}$ of mobile phase (acetonitrile 100 %).

Fig. 1 A flow chart representing the modified fractional crystallization method for extraction of squalene

Apparatus

The solvent delivery system consisted of Hittachi La Chrome Elite HPLC system equipped with a Diode Array Detector (Hitachi L-2455), a pump with an in-built degasser (Hitachi L-2100/2130), Oven (Hitachi L-2300) and an autosampler (Hitachi L-2200). The software used for analysis was Hitachi D-2000 Elite.

Measurements

The RP-HPLC method was performed as described by Lu et al. (2003) with some modification like selection of an RP C₁₈ (250 mm×4.6 mm) column with a core shell technology (distinct peak separation and fast elution compared to conventional column). The injection volume was set to 30 µl and the temperature of the column was set to 35 °C for analysis. Acetonitrile (100 %) was used as mobile phase at a flow rate of 1.5 ml min⁻¹ in an isocratic mode and the program was set for 30 min. Analytes were monitored with a diode array detector at a wavelength of 195 nm.

Results

In general the aim of our study was to establish a simple and rapid method for simultaneous analysis of squalene and cholesterol. Ten Indian fresh water and marine fish species were evaluated for their squalene and cholesterol content in different body parts like head, meat, fins and viscera. The optimum chromatographic conditions for determination of squalene were achieved with 100 % acetonitrile. Analytes were eluted

isocratically at a flow rate of 1.5 ml min⁻¹ with the injection volume of 30 µl using diode array detection at 195 nm. A typical HPLC chromatogram of fractional crystallized oil sample of mackerel meat is depicted in Fig. 2. The elution pattern for other fish samples obtained was in the similar manner where cholesterol and squalene were eluted at 11th and 14.2nd min respectively. This difference in retention time was sufficient enough to observe clear distinguished peaks. The quantitative values of squalene in different body parts of fresh water and marine fish samples have been illustrated in the Fig. 3a and b respectively. The Measurement was taken in terms of area of the peak and a ratio was established between squalene and cholesterol content. Simultaneous determination was possible as both squalene and cholesterol, were soluble in ethanol during fractional crystallization. Although interference was present in a few samples, they did not affect the separation and analysis of squalene and cholesterol. This analytical protocol was also followed for the analysis of squalene in olive oil, amaranth seed oil and RB DOD, where a clearly separated peak for squalene was observed for these samples as well, as depicted in Fig. 4a, b and c respectively.

This analytical data obtained was significant enough to establish a correlation between cholesterol and squalene for the different body parts of fish species used in our study as shown in Table 1 where the average ratio was found to be ranging from 2 to 10. For every 8 to 10 parts of cholesterol 1 part of squalene was found in the head and meat, whereas 6 to 10 parts of cholesterol was found for 1 part of squalene in the processing wastes like viscera and fins. The individual ratios deduced for different body parts of cholesterol and squalene content in fresh water and marine fish species have been mentioned in Table 1.

Calibration

Chromatographic peaks of squalene and cholesterol from the samples were identified based on the retention times of commercial standards. Peak identities were confirmed by spiking samples with standards and also on the characteristic UV-spectrum of each substance, with or without standard co-elution. For quantitative determination, squalene and cholesterol were monitored at 195 nm.

Method validation

Selectivity

The chosen chromatographic condition proved to be adequate for the separation of cholesterol and squalene in fish oil samples. The methods selectivity was confirmed using the Hitachi D-2000 Elite software, showing good resolution of the peak confirming that both these compounds were not co-eluted.

Fig. 2 A representative chromatogram depicting squalene & cholesterol peak in Mackerel meat analyzed through RP-HPLC. The conditions for HPLC analysis have been described in method section

Linearity

A standard calibration curve was established by plotting peak area against concentration, by using different concentrations of squalene and cholesterol as described in method. An excellent linearity was observed for the concentration curve. Further the coefficients of determination was found to be superior to 0.999 for all the curves, which confirmed that the

method generated proportional results to the concentrations of the analytes, within a specific range, being possible to correlate areas and analytes concentrations.

Repeatability and intermediary precision

Five replicates of the above mentioned sampled were prepared and analyzed by RP-HPLC. Each sample was analyzed twice

Fig. 3 Squalene content in different body parts expressed in $\mu\text{g mL}^{-1}$ of fish oil: **a** Commercial fresh water fish, **b** marine fish

Fig. 4 Chromatogram of squalene extracted from: **a** Olive oil, **b** Amaranthus grain, **c** Deodorized distillate of rice bran oil

and for every two samples a standard including both squalene and cholesterol at concentrations 50 and 400 $\mu\text{g ml}^{-1}$ respectively were injected. A satisfactory value for the precision of the method was obtained ($\text{CV}\%=3.05$). A CV % of 4.24 was obtained for squalene standard solution for measurement precision.

Recovery

To determine the recovery of the procedure two levels of standard squalene solution added were used for the recovery studies and each level of fractional crystallization was repeated five times. The recovery percentages for

Table 1 Ratio^a of cholesterol to squalene in different body parts of Indian fresh water & Marine fish ($n=6$)

	Ratio of cholesterol: Squalene (w/w)			
	Head	Meat	Viscera	Fins
<i>L rohita</i>	5.8	10.6	5.1	9.3
<i>C catla</i>	2.0	8.5	9.0	7.6
<i>C carpio</i>	4.5	7.3	9.5	8.4
<i>O mossambicus</i>	6.7	8.4	8.2	8.6
<i>C mrigala</i>	5.8	7.1	8.0	6.9
<i>N japonicus</i>	10.0	8.3	9.2	8.0
<i>S longiceps</i>	10.2	5.3	9.1	6.8
<i>E affinis</i>	7.3	5.2	8.9	6.3
<i>P pangasius</i>	1.6	5.3	4.1	2.8
<i>R kanagurta</i>	7.1	8.9	5.6	3.6

^a Values indicate parts of cholesterol for every part of squalene present in that body part

both the levels was found to be satisfactory (96.5 ± 5 and 95.2 ± 6).

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) for squalene was found to be 5.0 and 16.5 ng ml^{-1} and similarly for cholesterol the values were found to be 10.0 and $33.6 \text{ } \mu\text{g ml}^{-1}$ respectively for a signal to noise ratio of 3:1.

Discussion

The method described in the study offers various advantages over those reported in the literature. Firstly it was possible to simultaneously detect the two compounds squalene and cholesterol in a complex mixture (Fish oil), while methods described by previous authors enabled them to analyze either of the two compounds, squalene or cholesterol separately. Further, the method developed included a modified fractional crystallization technique where only a single solvent like absolute ethanol was used followed by incubation for 6 h at room temperature. This process successfully separated the two compounds from the sample and the supernatant was directly taken for analysis after the precipitate settled down. Fractional crystallization has been employed earlier, where a mixture of methanol/acetone (7:3 vol/vol) was used followed by incubation at -22°C (Nenadis and Tsimidou 2002). This method involved steps like filtration and vacuum evaporation prior to analysis which would lead to a considerable loss of the analyte. In the present method steps like cold storage, filtration and vacuum evaporation were eliminated as previously reported in the literature. It also provided better performance against

the classic saponification method for the following reasons. First, large number of samples can be analyzed as this method is quite simple and quick. Second, it is not necessary to subject the samples to oxidative reactions and high temperatures that may degrade the analyte which often happens in the case of saponification. Third, this method could be performed at room temperature and eliminated steps of vacuum evaporation thus providing a simple and rapid procedure. Lastly this method can also be relied upon for analysis of other sources of squalene apart from fish samples. Whereas in this paper a simplified fractional crystallization technique was employed and no further steps like filtration or evaporation were used, which resulted in minimal loss of analyte.

In previous reports HPLC analysis of squalene in olive oil was determined using a mixture of acetone/acetonitrile as mobile phase and UV detection at 208 nm (Nenadis and Tsimidou 2002). However acetone has high background absorption at this wavelength resulting in low sensitivity (Lu et al. 2004). Other previous reports suggest usage of methanol as mobile phase at 203 nm, which resulted in low background absorption and high detection sensitivity (Spanggaard et al. 2002). In our study, acetonitrile was used as the mobile phase which had background absorption lower than that of methanol or acetone/acetonitrile mixture. The detection sensitivity was also higher when compared to other reports (Lu et al. 2004). A C_{18} (5 μ) column with core shell technology was used instead of a conventional RP C_{18} column. Cholesterol and squalene were found to elute at a retention time of 28th & 32nd min respectively for conventional C_{18} columns with solid core particles whereas in C_{18} columns with core shell technology they were found to be eluting at 11th and 14.2nd min respectively. This significantly reduced the retention time of the analytes. With traditional fully porous particles, efficiency decreases as flow rate increases, resulting in loss of resolution and sensitivity, which slows down the overall analysis time. Core-shell technology enables high resolution and sensitivity over an extended linear velocity without generating excessive backpressure. This particle morphology results in less band broadening of sample peak in the chromatogram compared to fully porous particles and thus delivers extremely high efficiencies in the results.

Conclusion

Finally we would like to conclude that the present method showed satisfactory results with simple and rapid sample preparation by fractional crystallization method. Ethanol was found to be effective for separation of squalene and cholesterol from complex mixture in the different samples. Further a modified HPLC analysis using core shell technology offers significant improvements over previous published methods. This is a quick and reliable analytical procedure that makes

it possible to determine squalene and cholesterol simultaneously. This method was efficient enough to derive a ratio between these two compounds in different body parts of fish. Moreover this method was reliable for analysis of squalene in other rich sources without employing tedious conventional separation methods.

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