Available online at www.sciencedirect.com

SciVerse ScienceDirect

Procedia Environmental Sciences 15 (2012) 43 – 46

Procedia Environmental Sciences

The 1st Asia-Oceania Algae Innovation Summit (AOAIS-1)

Squalene quantification using octadecylbenzene as the internal standard

Hiroshi Matsuura*, Makoto M. Watanabe, Kunimitsu Kaya

Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8572, Japan

Abstract

Squalene was determined by HPLC using octadecylbenzene (ODB) as an internal standard. Squalene and ODB were monitored at 210 nm. The retention times of squalene and ODB were 7.59 and 8.54 min, respectively. Squalene was determined from the peak area ratios of squalene/ODB detected at 210 nm. After treatment with 0.5 M KOH containing ethanol at 90 °C for 1 h, squalene in the saponified lipid fraction was extracted using *n*-hexane. No interfering peak was observed. Linearity of this method was observed in the range 80−900 ng. ODB is useful as an internal standard for squalene determinations.

Keywords: squalene; octadecylbenzene; HPLC; *Aurantiochytrium*

1. Introduction

Squalene (SQU; **1**) is a triterpene hydrocarbon precursor for all animal and plant steroids, and is used in cosmetic industries [1], pharmaceuticals and medical sectors [2]. Many analytical methods have been reported for detecting SQU in various matrixes. For this investigation, we reviewed numerous published HPLC methods using C8 or C18 columns, organic mobile phases such as acetonitrile/acetone, and UV detection at wavelengths from 195 to 215 nm using SQU as an external standard [3-15]. We found that the quantified values are affected by the amounts of SQU recovered from the samples. For example, SQU recoveries were between 89.6% and 100.5% according to the determination in spiked samples [11]. However, the recovery tests are unnecessary when using an internal standard.

* Corresponding author. Tel.: +81-29-853-8812; fax: +82-29-853-8807.
E-mail address: h_matsuura@ies.life.tsukuba.ac.jp.

doi:10.1016/j.proenv.2012.05.008

To develop an accurate SQU determination method, we selected the non-biological hydrocarbon octadecylbenzene (ODB; **2**) for use as an internal standard. In this study, we report a method for accurately levels of SQU.

2. Materials and Methods

SQU samples were prepared from a culture of *Aurantiochytorium* sp. collected from the mangrove shore of Japan. SQU standard (99%) and octadecylbenzene (ODB, 98%) as an internal standard, were purchased from Wako Pure Chemicals (Osaka, Japan). Cells were maintained and grown in GPY medium (20 g glucose; 5 g yeast extract; 10 g polypeptone; 500 mL sea water; 500 mL freshwater). The cells were grown isothermally at 25 °C and were harvested during the early stationary phase. Cells were collected by centrifugation at 2500 rpm for 30 min, and then lyophilized after washing with distilled water.

To determine SQU levels in the cells, an aliquot of ODB (acetone solution) was added to the lyophilized cells, and lipids were extracted using chloroform/ methanol (2:1, v/v). The extract was evaporated under reduced pressure at 35°C. The remaining lipid was hydrolyzed using 0.5 M potassium hydroxide containing ethanol (0.5 M KOH/EtOH) at 90 °C for 1 h. 0.5 M KOH/EtOH was prepared as follows. KOH (2.805 g) was dissolved with a minimum amount of water. The KOH solution was diluted to 100 mL with EtOH. After saponification, non-saponifiable lipids were extracted with *n*-hexane. The extract was evaporated under reduced pressure at 37°C and redissolved with 1.0 mL of a mixed solvent of acetonitrile/tetrahydrofuran (THF) (9:1, v/v). The solution was automatically injected into an HPLC (Shimadzu LC-20) equipped with a photodiode array detector and a sample injector. Separation of analytes was conducted on a Mightysil RP-18 GP (150 ×2.0 mm; 5μm; Kanto Chemicals, Tokyo, Japan). The mobile phase consisted of acetonitrile/THF (80:20, v/v) at a flow rate of 0.2 mL/min, and ran under isocratic conditions. The sample injection volume was 1.0 μL, the column temperature was set at 30 °C, and the sample injector was maintained at 10°C. SQU and the internal standard (ODB) were detected at 210 nm.

Overall recoveries of SQU and ODB were examined using spiked samples of lyophilized cells. The spiked sample was prepared by addition of SQU (400 μg) and ODB (500 μg) to the lyophilized cells. The spiked and non-spiked samples were treated using the above procedure and the concentrations of SQU and ODB in spiked and non-spiked samples were determined by the peak area at 210 nm. SQU recovery was determined from the formula: Recovery = [(SQU detected in the spiked sample) – (SQU detected in the non-spiked sample)]/[spiked SQU (400 μg)]. ODB recovery was determined using the following formula: Recovery = (ODB detected in the spiked sample)/[spiked ODB (500 μg)]. Overall recoveries of SQU and ODB were 95.4 ± 2.3% and 96.6 ±1.8 % (n = 3), respectively.

3. Results and discussion

As shown in Fig. 1, SQU was eluted at a retention time (RT) of 7.59 min and ODB, the internal standard, was eluted at RT 8.58 min. Both peaks were completely separated. When different amounts of SQU and ODB were injected into the HPLC system, the peak areas of SQU increased in proportion to the injected amount from 80 to 900 ng, and those of ODB also increased proportionally, from 40 to 1200 ng. Identical amounts (500 ng) of SQU and ODB were injected into the HPLC system, and the peak area ratio of SQU /I.S (ODB) was found to be 2.31.

To confirm the linearity of the peak area ratio of SQU and ODB, SQU [0.20 (80 μg), 0.73 (300 μg), 1.22 (500 μg), 1.71 (700 μg), 2.20 (900 μg) and 2.44 μmol (1000 μg)] and ODB [1.51 μmol (500 μg)], which was used as an internal standard, were each dissolved in 1.0 mL of 10% THF containing acetonitrile solution, and the peak areas ratios of SQU/ODB in the each solution was expressed as the

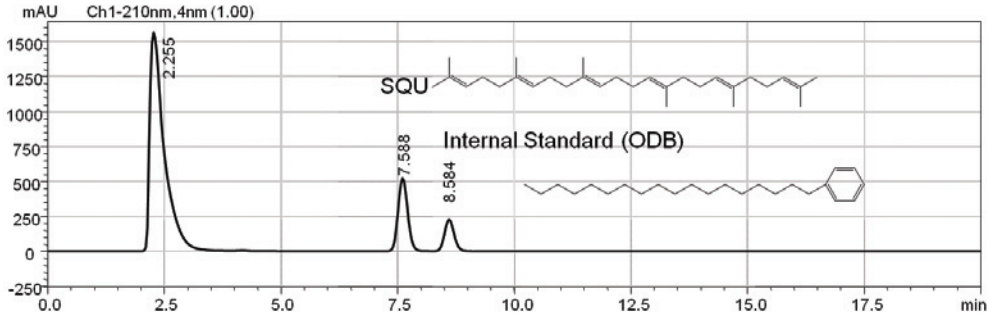

Fig. 1 Chromatogram of SQU [500 ng (1.22 nmol) /1.0 μL] and internal standard (ODB) [500 ng (1.51 nmol)/1.0 μL] obtained using HPLC with a Mightysil ODS column. SQU and ODB were monitored at 210 nm and eluted at 7.59 and 8.58 min, respectively.

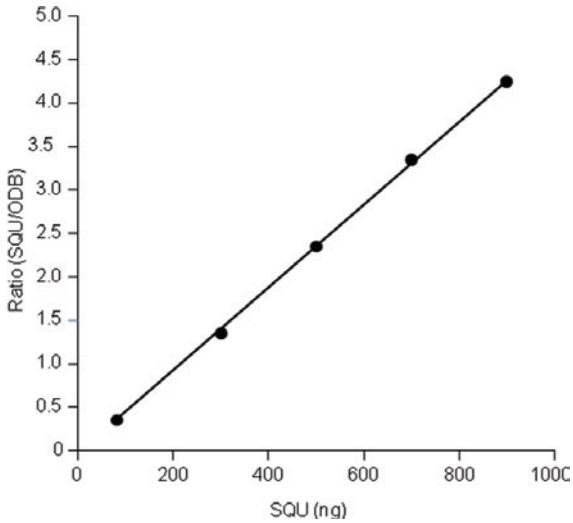

Fig. 2. Relationship between SQU concentration (80–900 ng) and the peak area ratio of SQU and ODB (internal standard). ODB [500 ng (1.51 nmol)] was added to SQU solution before treatment. After treatment, the sample volume was adjusted to 1.0 mL, and a fixed volume (1.0 μL) of sample solution was injected into the HPLC using an auto-injector. The liniear regression equation obtained was Y=0.0046X+0.003 (R= 0.9987), where X is the amount of SQU (ng), Y is the ratio of peak areas of SQU and ODB.

average of the four replicate measurements. Linearity between SQU concentration and the peak area ratio of SQU and ODB was confirmed to be in the range 0.20–2.20 μmol mL^{-1} (80–900 ng/1 μL injection volume). Calibration curves were constracted by plotting the corresponding peak area ratios of SQU/ODB versus the corresponding SQU concentration (Fig. 2). The limit of quantitation (S/N= 10) and the upper limit at 210 nm were 80 and 900 ng, respectively.

Generally, SQU recoveries from olive oil and algal samples were 89.6–100.5% [9]. Percentage recovery was determined using SQU-spiked samples. However, the recovery test is unnecessary when using of an internal standard. To date, an internal standard for SQU determination has not been used abecause a suitable compound for use as an internal standard has not been identified. ODB is a suitable compound for use as an internal standard, because ODB is a synthetic compound not found in natural

products, and the recovery of ODB as an internal standard is nearly the same as that of SQU. If mass spectrometry (MS) is used as a detector in this method, more sensitive analysis is possible.

Acknowledgement

This work was supported by the Japan Science and Technology Agency (JST)-CREST funding and the Special Funds for Next Generation Energy Development Project, MEXT, Japan.

References

[1] Aioi A, Shimizu T, Kuriyama K. Effect of squalene on superoxide anion generation induced by a skin irritant, lauroylsarcosine. *Int J Pharm* 1995;**113**:159-64.

[2] World Health Organization, Global Advisory Committee on Vaccine Safety, (http://www.sho.int/vaccine_safety/topics/adjuvants/squalene/questions_and_answers/en/)

[3] Fujimoto N, Katai M, Meguro H. Determination of sterols in fat by high performance liquid chromatography. *BUNSEKI KAGAKU* 1986;**35**:482-6.

[4] Imaeda K, Ohsawa K, Watanabe T. Simultaneous determination of cholesterol and squalene in human skin surface lipids by HPLC. *BUNSEKI KAGAKU* 1987;**36**:T140-T143.

[5] Fliesler SJ, Florman R, Rapp LM, Pittler SJ, Keller RK. In vivo biosynthesis of cholesterol in the rat retina. *FEBS Lett* 1993;**335**:234-8.

[6] Grieveson LA, Ono T, Sakakibara J, Derrick JP, Dickinson JM, McMahon A, Higson SP, A simplified squalene epoxidase assay based on an HPLC separation and time-dependent UV/Visible determination of squalene. *Anal Biochem* 1997;**252**:19-23.

[7] Hagiwara T, Yasuno T, Funayama K, Suzuki S. Determination of lycopene, a-carotene and β-carotene in serum by liquid chromatography- atmospheric pressure chemical ionization mass spectrometry with selected-ion monitoring. *J Chromatography B* 1998;**708**:67-73.

[8] Villén J, Blanch GP, Castillo MLR, Herraiz M. Rapid and simultaneous analysis of free sterols, tocopherols, and squalene in edible oils by coupled reversed-phase liquid chromatography-gas chromatography. *J Agric Food Chem* 1998;**46**:1419-22.

[9] Lu HT, Jiang Y, Chen F. Preparative separation and purification of squalene from the microalga *Thraustochytrium* ATCC 26185 by high-speed counter-current chromatography. *J Chromatography A* 2003;**994**:37-43.

[10] Vidal-Escales E, Borrós S. New methodology to follow the evolution of squalene by-products during model compound vulcanization studies. *Talanta* 2004;**62**:539-47.

[11] Lu HT, Jiang Y, Chen F. Determination of squalene using high-performance liquid chromatography with diode array detection. *Chromatographia* 2004;**59**:367-71.

[12] Chua CSL, Baharin BS, Man YBC, Tan CP. Separation of squalene from palm fatty acid distillate using adsorption chromatography. *Eur J Lipid Sci Technol* 2007;**109**:1083-7.

[13] Grigoriadou D, Androulaki A, Psomiadou E, Tsimidou MZ. Solid phase extraction in the analysis of squalene and tocopherols in olive oil. *Food Chem* 2007;**105**:675-80.

[14] Chem G, Fan KW, Lu FP, Li Q, Aki T, Chen F, Jiang Y. Optimization of nitrogen source for enhanced production of squalene from thraustochytrid *Aurantiochytrium* sp. *New Biotechnol* 2010;**27**:382-9.

[15] Spanggord RJ, Wu B, Sun M, Lim P, Ellis WY. Development and application of an analytical method for the determination of squalene in formulations of anthrax vaccine adsorbed. *J Pharma Biomedic Analy* 2002;**29**:183-93.

Received 24 July 2011; accepted 3 September 2011