Chapter 7

Biosynthetic Approaches to Squalene Production: The Case of Yeast

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Abstract

Squalene is a precursor in the eukaryotic sterol biosynthesis. It is a valuable compound with several human health-related applications. Since the traditional natural resources of squalene are limited, alternatives for the production of squalene on industrial scale have been intensively explored during past years. The yeast *Saccharomyces cerevisiae* represents an attractive option due to elaborated techniques of genetic and metabolic engineering that can be applied to improve squalene yields. We discuss in this chapter some theoretical aspects of genetic manipulations of the ergosterol biosynthesis pathway aimed at increased squalene production and describe analytical methods for squalene purification and determination of its content in yeast cells.

Key words Squalene, Yeast, Ergosterol biosynthesis, Squalene monooxygenase, *ERG1* gene, Lipid extraction, Solid phase extraction, Thin-layer chromatography, High-performance liquid chromatography

1 Introduction

Squalene is a linear polyunsaturated triterpenoid with many applications in cosmetic and pharmaceutical industry (e.g., as a vaccine adjuvant component), or as a nutritional supplement. Although it is synthesized in many organisms, its natural sources are limited. The richest available source of squalene is shark liver oil; however, the long-term dependence of squalene production on this oil may not be feasible due to environmental concerns, such as excessive fishing of sharks or increasing contamination by pollutants. Alternative natural sources explored in recent years include plants (e.g., olives or amaranth) or microorganisms [1]. Yeasts represent in this respect a very promising alternative option for squalene production. High levels of squalene have been found in some natural isolates of the genus *Pseudozyma* [2], but the biotechnologically established yeast *Saccharomyces cerevisiae* can be genetically engineered for high production of squalene.

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Squalene is synthesized in *S. cerevisiae* in the mevalonate pathway as the first metabolite dedicated to ergosterol biosynthesis [3]. Its physiological levels are typically very low due to the rapid utilization for ergosterol biosynthesis. Generally, accumulation of squalene can be reached either by increasing the production in the mevalonate pathway, or by reducing the consumption in the post-squalene part of ergosterol biosynthesis. Both approaches have been successfully applied to increase squalene levels in yeast.

There are two enzymes in the mevalonate pathway potentially suitable for increasing squalene levels: HMG-CoA reductase (encoded in two homologous genes *HMG1* and *HMG2*) and squalene synthase (encoded in *ERG9* gene), but only the first one has been proved as an efficient target. HMG-CoA reductase is one of the major control sites of eukaryotic sterol biosynthesis. Yeast *S. cerevisiae* has two isoforms of HMG-CoA reductase: Hmg1p representing the major aerobic activity and Hmg2p expressed preferentially during hypoxia [4]. Simple overexpression of the *HMG1* gene has only limited effect on squalene levels due to the tight control of Hmg1p activity [5] and due to the detrimental effect of the formation of the ER membrane stacks ("carmellae") in cells overproducing Hmg1p [6]. These limitations have been overcome by expressing a truncated form of Hmg1p that lacks ER membrane targeting domain and retains the functional catalytic domain. Expression of this truncated Hmg1p under strong promoters caused significant accumulation of squalene in yeast cells [7–9].

An alternative approach to increase cellular squalene is to limit squalene consumption in the post-squalene part of ergosterol biosynthesis. It must be emphasized that not all enzymes in the pathway are suitable for the genetic modification of squalene levels. Very promising in this respect is squalene monooxygenase catalyzing squalene epoxidation as the first step in squalene metabolic transformation. This enzyme is coded by the *ERG1* gene in *S. cerevisiae* [10] and it is the target for the antimycotic terbinafine commonly used to treat dermatomycoses [11]. Terbinafine at subinhibitory concentrations has been shown to increase very efficiently squalene levels in *Saccharomyces cerevisiae* [12–14] and in *Kluyveromyces lactis* [15]. However, use of this antimycotic for squalene production has several drawbacks, e.g., high economic costs, or loss of yeast cell viability at terbinafine concentrations yielding high squalene levels [14].

During the mutational mapping of the squalene monooxygenase we have engineered a set of single-point mutants in the *ERG1* gene either resistant or hypersensitive to terbinafine [16–18]. The hypersensitive mutants turned out to be very interesting with respect to squalene production. They retained low squalene monoxygenase activity (that was nevertheless sufficient to maintain cell growth) and accumulated high levels of squalene [14, 18]. A spontaneous mutation in the *ERG1* gene isolated as the suppressor

of *erg26ts* allele [19] showed similar characteristics as the engineered strains. Moreover, all above mentioned *erg1* mutants accumulated squalene without compromising cell growth and viability. This proves that targeting squalene monooxygenase by mutations of the *ERG1* gene is generally suitable as a tool for the biotechnological production of squalene in yeast.

This chapter describes the basic principles of construction of squalene-accumulating mutants in the *ERG1* gene with reduced activity of squalene monooxygenase. We present here detailed laboratory protocols for the isolation of non-saponifiable lipids (sterols and squalene) from yeast cells, purification of squalene from these lipid extracts by solid phase extraction (SPE) and two chromatographic methods for qualitative and quantitative analysis of squalene and sterols in lipid extracts (thin layer chromatography, TLC, and high-performance liquid chromatography, HPLC) that can be performed in laboratories with standard biochemical equipment. The SPE method described here can be used for the isolation of purified squalene in milligram quantities as well as for the isolation of radiolabeled squalene and ergosterol from yeast cells grown in media supplemented with ^{14}C-acetate.

2 Materials

2.1 Yeast Strains and Isolation of erg1 Mutants

The following *S. cerevisiae* strains were used in the experiments:

1. Wild-type strain W303-1B [genotype *MATα*, *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 can1-100*].
2. *erg 1* mutant strain [genotype *MATa leu2 ura3 trp1 ERG1*::*URA3* (transformed with plasmid pNS1 containing the *erg1L37P* allele under own promoter)].

Isolation of erg1 Mutant

erg1L37P mutation causing L37P substitution in the FAD-binding domain of the Erg1p was generated by in vitro random mutagenesis via PCR amplification as described in [18]. Briefly, *ERG1* gene was amplified from a recombinant plasmid carrying the wild-type *ERG1*gene using the forward primer (5′-ACGACGTTGTAAAAC GACGGCCAG-3′) and the reverse primer (5′-TTCACACAGG AAACAGCTATGACC-3′). The amplification was performed with DyNAzyme II DNA polymerase (Finnzymes) in the presence of 5 mM $MgCl_2$ and 0.05 mM $MnCl_2$. PCR fragments were digested with *Pst*I, cloned into the centromere vector pRS315, and transformed into *E. coli* XL1. Plasmid DNA was isolated from *E. coli* by standard procedures [20] and transformed into *S. cerevisiae* KLN1 strain (containing the disruption of chromosomal *ERG1*::*URA3*, *see* **Note 1**) by the protocol of Gietz et al. [21]. Clones expressing a functional squalene epoxidase were selected by complementation

of the aerobic growth defect of KLN1 *ERG1*::*URA3* on YPD agar plates. Positive clones were tested for terbinafine sensitivity on YPD plates containing 10 μg/ml of terbinafine. Plasmid DNA was isolated from terbinafine-hypersensitive *S. cerevisiae* clones [22], amplified in *E. coli* XL1 and characterized by DNA sequencing.

2.2 Equipment

1. FastPrep®-24 cell homogenizer (MP Biomedicals) with adapter for 15-ml tubes (12 × 15-ml TeenPrep™ adapter).
2. Sample concentrator (Stuart) connected to the source of nitrogen (high purity nitrogen in pressure gas cylinder).
3. Semi-automatic sample applicator Linomat 5 (CAMAG).
4. Glass microsyringes with volumes 25, 100, and 500 μl (Hamilton).
5. Three TLC developing chambers (Twin Trough chamber CAMAG with glass lid, 20 × 20 cm).
6. HPLC instrument (Agilent 1100) equipped with reversed phase C8 column (Eclipse XDB-C8, particle size 5 μm, column size 4.6 × 150 mm, Agilent), diode array detector (Agilent 1100), Corona® Charged Aerosol Detector (ESA/Thermo Scientific), and nitrogen generator (ESA/Thermo Scientific).

2.3 Reagents and Supplies

1. YEPD rich growth medium (1 % yeast extract, 2 % peptone, 2 % dextrose).
2. 15-ml polypropylene tubes.
3. 1.5-ml microcentrifuge tubes.
4. Acid-washed glass beads, diameter 0.4–0.6 mm (Sigma-Aldrich) (*see* **Note 2**).
5. 20 and 12 ml Pyrex® glass tubes with Teflon-lined cups (Corning) (*see* **Note 2**).
6. Methanolic KOH solution with pyrogallol (60 % KOH w/v, 50 % methanol v/v, 0.5 % pyrogallol w/v) (*see* **Note 3**).
7. Organic solvents: *n*-hexane, methanol, diethylether, petroleum ether (boiling point 60–80 °C), chloroform (all of the highest purity available, *see* **Note 2**).
8. Water (LiChrosolv®, Merck Millipore).
9. Glacial acetic acid (p.a. EMSURE®, Merck Millipore).
10. TLC silica gel 60 aluminum sheets (20 × 20 cm, Merck Millipore).
11. SPE column CHROMABOND® NH2 (3 ml, 500 mg, Macherey-Nagel).
12. TLC solvent developing mixture I: petroleum ether–diethyl ether–acetic acid (35:15:1, v/v).
13. TLC solvent developing mixture II: petroleum ether–diethyl ether (49:1, v/v).

14. TLC charring solution: 0.63 g $MnCl_2.4H_2O$, 60 ml water, 60 ml methanol, 4 ml sulfuric acid (p.a., 96%).
15. HPLC standard ergosterol (Sigma-Aldrich Fluka, purity ≥95%); 1 mg/ml stock solution in ethanol.
16. HPLC standard squalene (Sigma-Aldrich, purity ≥98%); 1 mg/ml stock solution in ethanol.

3 Methods

3.1 Extraction of Non-saponifiable Lipids

1. Grow yeast cells in a rich YEPD medium to late exponential/early stationary phase (16–20 h) (*see* **Note 4**).
2. Harvest the cells by centrifugation at 700 × *g* for 5 min and discard supernatant.
3. Wash the sediment with deionized water and harvest the cells by centrifugation (700 × *g*, 5 min).
4. Resuspend sediment in deionized water to a concentration of 1×10^9 cells/ml. Transfer 1 ml aliquot of cell suspension into a new polypropylene 15-ml tube.
5. Add approx. one volume (1 ml) of glass beads (measured in a calibrated 1.5 ml microcentrifuge tube) to the tube and cool on ice.
6. Break the cells in a FastPrep24 homogenizer 2 × 45 s (6.5 m/s speed) with 5 min cooling on ice between the breaking runs (*see* **Note 5**).
7. Transfer broken cell suspension with a glass Pasteur pipette to 20 ml Pyrex glass tube with Teflon-lined cup. Be careful to avoid the carryover of glass beads.
8. Add three volumes (3 ml) of methanolic KOH with pyrogallol and incubate for 2 h at 70 °C (*see* **Note 6**).
9. Cool the saponification mixture, add three volumes (3 ml) of *n*-hexane and mix well on a vortex mixer.
10. Separate the organic and water phases by centrifugation for 5 min at 1300 × *g*.
11. Transfer the upper organic phase to a clean 12 ml Pyrex glass tube with Teflon-lined cup.
12. Re-extract the water phase with three volumes (3 ml) of *n*-hexane, collect the upper phase separated by centrifugation (as in **step 10** of Subheading 3.1) and join both organic phases.
13. Evaporate *n*-hexane from the joined organic phases in sample concentrator under the stream of nitrogen.
14. Dissolve the lipid residue containing squalene and sterols in desired volume of solvent (e.g., *n*-hexane), and store at −20 °C in 2 ml amber glass vial with Teflon-lined cup.

3.2 Semi-preparative Separation of Squalene and Sterols by Solid Phase Extraction (SPE)

1. Prepare the SPE column by conditioning three times with 3 ml of *n*-hexane (*see* **Note 7**).
2. Load 3 ml of the non-saponifiable lipid extract in *n*-hexane on the top of the column (*see* **Note 8**).
3. Collect the flow-through.
4. Elute squalene from the column two times with 3 ml of *n*-hexane and collect the eluates in the tube with the flow-through.
5. Evaporate the solvent from combined flow-through and two *n*-hexane eluates under the nitrogen stream and dissolve the lipid residue (containing squalene) in desired volume of solvent (e.g., *n*-hexane).

3.3 Two-Step TLC Separation of Squalene and Sterols [23] (See Note 9)

1. Load sample on the TLC plate using sample applicator or manually by glass microsyringe (*see* **Notes 10** and **11**).
2. Put the TLC plate with loaded samples in the preconditioned chamber with the developing solvent mixture I (petroleum ether–diethyl ether–acetic acid, 35:15:1 v/v) and develop until the solvent front reaches 5 cm from the top of the plate (approx. 30 min) (*see* **Note 12**).
3. Take out the TLC plate and evaporate the developing solvent mixture in a fume hood.
4. Transfer the TLC plate to the chamber preconditioned with the developing solvent mixture II (petroleum ether–diethyl ether, 49:1 v/v) and develop until the solvent front reaches about 1 cm from the top of the plate (approx. 45 min).
5. Take out the TLC plate and evaporate the developing solvent mixture in a fume hood.
6. Dip the plate into the charring solution for 0.5–1 min (*see* **Note 13**). Let the TLC plate dry in a fume hood.
7. Incubate the TLC plate at 130 °C in the oven for 10–20 min. Inspect periodically the appearance of dark brown lipid spots during this time period (*see* Fig. 1).

3.4 HPLC Separation of Squalene and Sterols

1. For HPLC analysis of non-saponifiable lipids (squalene and sterols), dry lipid extract equivalent of 1×10^9 cells and dissolve the residue in 300 µl of solvent (e.g., *n*-hexane or acetone) (*see* **Note 10**).
2. Load 10 µl aliquot (equivalent to $3–6 \times 10^7$ cells) on the C8 column using automatic sampler. Lipids are separated with 95 % methanol as the mobile phase. If applicable, column temperature is set to 30 °C (*see* **Note 14**).
3. Lipids are detected using two detectors connected in series. First, lipid peaks are detected based on their UV spectrum in a non-destructive diode array detector (DAD) followed by detection in the Corona Charged Aerosol Detector (CAD) (*see* **Note 15**) (*see* Fig. 2).

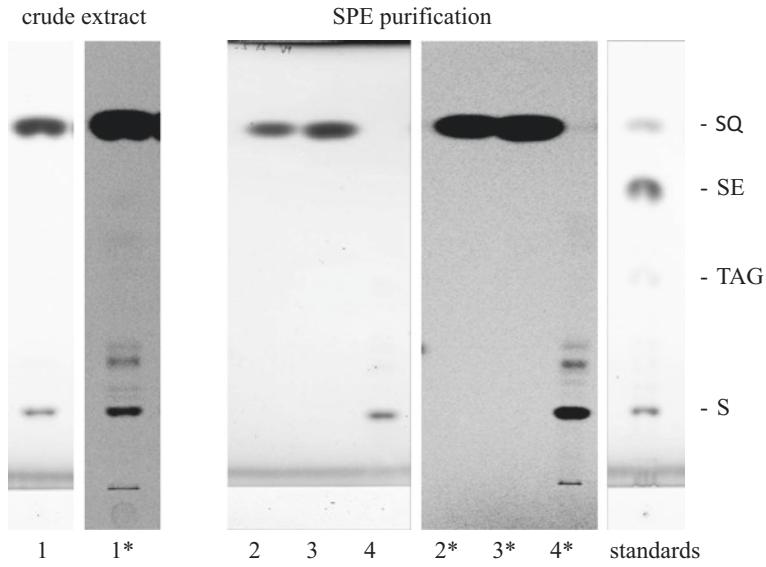
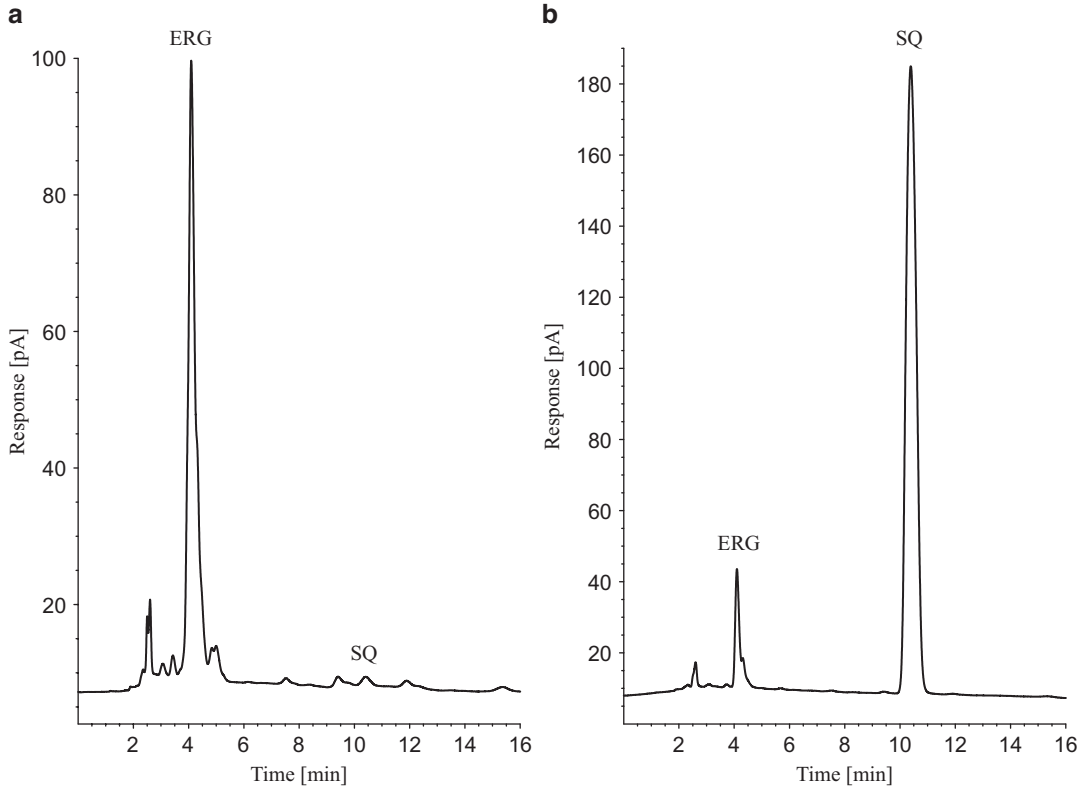


Fig. 1 TLC analysis of squalene purified from the non-saponifiable lipid extract on SPE column. *erg1*^{L37P} cells were grown for 16 h in YPD containing ^{14}C-acetate to label total lipids. Radioactivity on TLC plates was visualized by phosphorimager (*lanes with asterisks*) followed by charring with sulphuric acid (*see* 2.3.3.6) (*lanes w/o asterisks*). *1*: total non-saponifiable lipid extract (equivalent of 3×10^8 cells); *2*: purified squalene (equivalent of 1×10^8 cells); *3*: purified squalene (equivalent of 2×10^8 cells); *4*: methanol eluate of sterols remaining after *n*-hexane elution (equivalent of 3×10^8 cells); Standards: *SQ* squalene, *SE* cholesteryl oleate, *TAG* triolein, *S* sterols

4 Notes

1. Selection of *erg1* mutations with reduced squalene monooxygenase activity is performed in strains with disrupted chromosomal copy of *ERG1* gene. Such disruption is lethal under standard aerobic conditions; however, disruptant strain can be cultivated anaerobically in YPD media supplemented with 20 μg/ml of ergosterol and 0.06 % Tween 80 (as the source of unsaturated fatty acids).
2. All organic solvents used in lipid extraction and chromatographic separation should be of highest purity. Merck LiChrosolv®, EMPLURA®, or EMSURE® quality solvents were tested with satisfactory results and are recommended. Plastic material (tips, containers, etc.) must not be used during the extraction procedure (with exception of the cell breaking step). Organic solvents release additives from the plastics which might interfere with subsequent analysis. Glass and Teflon® materials should be used throughout the procedure.
3. To prepare 10 ml of methanolic KOH solution it is recommended to dissolve 6 g of KOH pellets in 5 ml of 1 % pyrogallol



	ERG	SQ
ng/peak	1700	96
µg/10^9 cells	51	3

	ERG	SQ
ng/peak	379	7932
µg/10^9 cells	11	238

Fig. 2 HPLC-CAD chromatogram of non-saponifiable lipid extract from wild-type (**a**) and *erg1*^{L37P} mutant (**b**). Lipids isolated from 1×10^9 cells were resuspended in 300 µl of hexane and 10 µl aliquots were separated by reversed-phase HPLC. Ergosterol (ERG) and squalene (SQ) were quantified as described in **Note 16**. Low amount of ergosterol in *erg1*^{L37P} mutant compared to the wild-type is caused by the absence of steryl esters due to reduced ergosterol biosynthesis

in methanol (w/v) mixed with 2.5 ml of water. When KOH pellets are fully dissolved, final volume should be adjusted to 10 ml with water. The reaction is strongly exothermic thus special care should be taken during preparation of the solution.

4. Start the culture with fresh (up to 24 h old) inoculum. Starting concentration should be in the range $0.5–1 \times 10^6$ cells/ml. Squalene levels in *erg1* mutants may decrease in the late stationary phase due to the utilization of accumulated squalene for ergosterol synthesis. It is therefore recommended to collect cells not later than 16–20 h. 40 ml of the culture of *erg1L37P* mutant yields approx. 1 mg of squalene.

5. Due to a rigid cell wall it is essential to break yeast cells for efficient extraction of squalene (and other neutral lipids stored in lipid droplets). If a dedicated homogenizer is not available, cells can be broken in 20 ml Pyrex tubes by vortexing with glass beads on a high-speed vortex. Six one-minute breaking intervals with 1 min cooling on ice between each round are recommended. In such a case **step** 7 of Subheading 3.1 is omitted and methanolic KOH mixture can be added directly to homogenate.
6. Efficiency of neutral lipid hydrolysis depends on the quality of methanolic KOH mixture. It is therefore strongly recommended to use freshly prepared solution and to adhere to the KOH concentration of 60%. The efficiency of saponification (steryl ester hydrolysis) should be checked on TLC, particularly if isolated lipids will be used for further squalene purification on SPE.
7. Vacuum manifold can be used to speed up the purification process. If the manifold is not available, elution can be driven simply by the gravitation force.
8. Macherey-Nagel claims their silica based CHROMABOND columns has the binding capacity approx. 3–5% of the amount of the adsorbent. For *erg1L37P* cells the lipid amount equivalent to the cell culture of approx. 500 ml (450–750 ml) can be loaded on the CHROMABOND NH2 (500 mg) column. If other yeast strains or other SPE columns are used, amounts of total isolated non-saponifiable lipids (squalene and sterols) should be determined before loading on SPE column.
9. Separation in a single solvent mixture (petroleum ether–diethyl ether–acetic acid; 35:15:1, v/v) can be used as well; however, it is recommended to perform two-step procedure to achieve good separation of squalene from possible steryl ester contaminants. It is recommended to use separate chambers for each solvent mixture.
10. Due to the high volatility of the solvents, lipid extracts are always dried under the stream of nitrogen and dissolved in an exact volume of the solvent immediately before chromatographic analysis.
11. Set up the sample applicator or load samples manually on the horizontal lines 2 cm from the bottom of the plate. Application spots should be 1.5 cm from the side edges of the plate with 1 cm distance between neighboring spots. Loading spot should be 1 cm long for loading lipids equivalent to 3×10^8 cells (dissolved in organic solvent of volume up to 30 μl). It is recommended to label marks 5 and 1 cm from the top of the plate at both side edges of the plate with soft pencil for the control of the development path in both developing steps.

12. It is essential to precondition the chamber with the developing mixture for at least 30 min with closed lid before putting in the TLC plate. Preconditioning can be improved by lining the walls of the chamber with thick filtration paper. The amount of the solvent used depends on the size of the chamber; however, the level of the solvent should be 1–1.5 cm from the bottom.
13. A developing chamber is used for charring solution. Large Petri dish that will fit 20×20 cm TLC plate can be used instead. If the TLC plate will be used for a preparative analysis of lipids, non-destructive staining of lipids with iodine should be used. The developed plate is then exposed to iodine vapors in the desiccator and temporary visible yellow lipid spots are labeled with a soft pencil.
14. Sterols present in the non-saponifiable lipid extracts originate from the free sterol fraction and from hydrolyzed steryl ester fraction. Squalene peak is well separated from sterols and elutes on XDB, C8 column size 4.6×150 mm with 5 μm particle size at mobile phase flow rate of 1 ml/min approximately 6 min after sterols. Similar results can be achieved with shorter columns as well as with C18 columns. If HPLC analysis is not used for preparative squalene purification, column temperature can be increased to 40 °C to speed up the separation process.
15. Retention time of squalene standard is used to identify squalene in analyzed samples. In DAD spectral analysis, lipids (including squalene) are detected in the wavelength range 200–220 nm. Some sterol structures have characteristic absorbance peaks outside this range (e.g., ergosterol at 260–300 nm). Be aware of different molar extinction coefficients of individual lipids if you plan to apply UV or DAD detectors for sterol or squalene quantification. If available, Corona CAD detector is highly recommended for lipid analysis. This detector is suitable for absorption spectrum-independent quantification of various analytes, including phospholipids, squalene and sterols. The detector is usually set to sensitivity 200 pA.
16. Ergosterol and squalene quantities were calculated using the following equations:

$$\text{ergosterol}[\text{ng}] = 2E-06 \times \mathbf{x}^2 + 0.089 \times \mathbf{x}, \text{where } \mathbf{x} \text{ is the area of the ergosterol peak}$$

$$\text{squalene}[\text{ng}] = 7E-07 \times \mathbf{x}^2 + 0.1391 \times \mathbf{x}, \text{where } \mathbf{x} \text{ is the area of the squalene peak.}$$

The equations represent second-order polynomial functions that were derived from the calibration curves for ergosterol and squalene analyzed on the Corona CAD detector. The calibration curves were constructed by measuring three independently weighed ergosterol or squalene standard dilution sets (0.02, 0.05, 0.1, 0.2, 0.4, 1.0 mg/ml.

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References

1. Spanova M, Daum G (2011) Squalene—biochemistry, molecular biology, process biotechnology, and applications. Eur J Lipid Sci Technol 113:1299–1320
2. Chang MH, Kim HJ, Jahng KY, Hong SC (2008) The isolation and characterization of *Pseudozyma* sp. JCC 207, a novel producer of squalene. Appl Microbiol Biotechnol 78:963–972
3. Daum G, Lees N, Bard M, Dickson R (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast 14:1471–1510
4. Basson ME, Thorsness M, Rine J (1986) *Saccharomyces cerevisiae* contains 2 functional genes encoding 3-hydroxy-3-methylglutaryl coenzyme-A reductase. Proc Natl Acad Sci U S A 83:5563–5567
5. Burg JS, Espenshade PJ (2011) Regulation of HMG-CoA reductase in mammals and yeast. Prog Lipid Res 50:403–410
6. Wright R, Basson M, D'Ari L, Rine J (1988) Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. J Cell Biol 107:101–114
7. Donald KA, Hampton RY, Fritz IB (1997) Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme a reductase on squalene synthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 63:3341–3344
8. Polakowski T, Stahl U, Lang C (1998) Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol 49:66–71
9. Asadollahi MA, Maury J, Schalk M, Clark A, Nielsen J (2010) Enhancement of farnesyl diphosphate pool as direct precursor of sesquiterpenes through metabolic engineering of the mevalonate pathway in *Saccharomyces cerevisiae*. Biotechnol Bioeng 106:86–96
10. Jandrositz A, Turnowsky F, Hogenauer G (1991) The gene encoding squalene epoxidase from *Saccharomyces cerevisiae*: cloning and characterization. Gene 107:155–160
11. Ryder NS (1992) Terbinafine: mode of action and properties of the squalene epoxidase inhibition. Br J Dermatol 126(Suppl):2–7
12. Naziri E, Mantzouridou F, Tsimidou MZ (2011) Enhanced squalene production by wild-type *Saccharomyces cerevisiae* strains using safe chemical means. J Agric Food Chem 59:9980–9989
13. Ta MT, Kapterian TS, Fei W, Du X, Brown AJ, Dawes IW, Yang H (2012) Accumulation of squalene is associated with the clustering of lipid droplets. FEBS J 279:4231–4244
14. Garaiová M, Zambojová V, Šimová Z, Griač P, Hapala I (2014) Squalene epoxidase as a target for manipulation of squalene levels in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 14:1567–1364
15. Drozdíková E, Garaiová M, Csáky Z, Obernauerová M, Hapala I (2015) Production of squalene by lactose-fermenting yeast *Kluyveromyces lactis* with reduced squalene epoxidase activity. Lett Appl Microbiol 61:77–84
16. Klobučníková V, Kohút P, Leber R, Fuchsbichler S, Schweighofer N, Turnowsky F, Hapala I (2003) Terbinafine resistance in a pleiotropic yeast mutant is caused by a single point mutation in the ERG1 gene. Biochem Biophys Res Commun 309:666–671
17. Leber R, Fuchsbichler S, Klobučníková V, Schweighofer N, Pitters E, Wohlfarter K, Lederer M, Landl K, Hapala I, Turnowsky F (2003) Molecular mechanism of resistance to terbinafine in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother 47:3890–3900
18. Ruckenstuhl C, Lang S, Poschenel A, Eidenberger A, Baral PK, Kohut P, Hapala I, Gruber K, Turnowsky F (2007) Characterization of squalene epoxidase of *Saccharomyces cerevisiae* applying terbinafine sensitive variants. Antimicrob Agents Chemother 51:275–284
19. Germann M, Gallo C, Donahue T, Shirzadi R, Stukey J, Lang S, Ruckenstuhl C, Oliaro-Bosso S, McDonough V, Turnowsky F, Balliano G, Nickels JT Jr (2005) Characterizing

sterol defect suppressors uncovers a novel transcriptional signaling pathway regulating zymosterol biosynthesis. J Biol Chem 280:35904–35913

20. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360
22. Strathern JN, Higgins DR (1991) Recovery of plasmids from yeast into *Escherichia coli*: shuttle vectors. Methods Enzymol 194:319–329
23. Spanova M, Czabany T, Zellnig G, Leitner E, Hapala I, Daum G (2010) Effect of lipid particle biogenesis on the subcellular distribution of squalene in the yeast *Saccharomyces cerevisiae*. J Biol Chem 285:6127–6133