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Recovery of Squalene from Wine Lees Using Ultrasound Assisted Extraction—A Feasibility Study

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Supporting Information

ABSTRACT: The present work is a systematic approach for valorization of wine lees regarding the recovery of squalene, a bioactive lipid. Such a study is presented for the first time in literature. Separate examination of squalene content in "light" and "heavy" lees from different vinification processes by RP-HPLC demonstrated that these waste streams can be used as a source for this lipid, despite variations due to technological or genetic effects. Next, ultrasound assisted extraction of squalene from the "industrial waste" (the mixture of wine lees generated from different wines) using *n*-hexane was optimized with the aid of response surface methodology (independent variables: sonication duration and duty cycles). Autolysis was monitored through optical microscopy. Squalene yield (0.6 ± 0.08 g SQ/kg dry lees) was comparable to that of recently examined potential sources (0.2−0.35 g SQ/kg dry olive pomace and 0.06 g SQ/kg olive leaves).

KEYWORDS: *squalene, wine lees, ultrasound assisted extraction, response surface methodology*

INTRODUCTION

Wine industries produce large volumes of wastes and byproduct including grape pomace, stalk, dewatered slush, and lees.[1] Winemaking process (white, rose, or red wines), results in the formation of "heavy" and "light" lees from the different decanting steps of wine. Although variable in composition, they are mainly composed of yeast cells and tartaric acid.[2,3] The high quantities of lees became the raw material for the commercial production of tartaric acid and ethanol.[3] Potential exploitation of this waste has also been proposed for the recovery of high value products and as a nutritional supplement for microorganisms.[3−8] Recent economic and environmental analyses of this winery waste upgrading[8] strengthen the prospect of implementing the above proposals and also inventing new applications.

A bioactive compound, appreciated in the food, pharmaceutical, and cosmetics industries that could potentially be recovered from wine lees is squalene. This biomass attracted our interest considering that a decrease in the sterol content of lees from Chardonnay winemaking under limited oxygen supply led to a concomitant increase in squalene content.[9] The finding, in line with our efforts to enhance accumulation of squalene in the cells of food grade strains of *S. cerevisiae* in the expense of ergosterol formation,[10−12] was the driving force to carry out experiments for its recovery directly from the waste material. The interest in establishing novel sources for squalene is increasing as the conventional ones are rather limited.[13]

Most research on the exploitation of wine lees focuses on the optimization of the recovery process for target compounds. Among others, recovery is highly dependent on the localization of the latter (intracellular, cell surface, or extracellular), a critical factor for the selection of suitable extraction means (chemical, enzymic, or mechanical treatments).[14,15] Regarding intracellular compounds, the need to destruct cell walls to achieve efficient penetration of the solvent is obvious. This is the case of squalene, found in the lipophilic core of lipid globules within the cells that are surrounded by a thick, rigid cell wall.[16,17] Its lysis facilitates to a high degree the entrance of nonpolar solvents, which are suitable for the extraction of squalene. Until now, there has been no literature report on an effective method for the selective extraction of squalene from wine lees. In the few articles that focus on the total lipid recovery, the employed protocols can be justified only for analytical purposes and not for food applications.[9,18]

To fulfill the aim of the present study, ultrasound assisted extraction (UAE), presenting the potential of scale-up, was selected as an appropriate procedure for lipid extraction from solid matrices.[19−21] Safety aspects limited alternatives among permitted solvents for food applications.[22] Thus, *n*-hexane, a solvent used in the production or fractionation of fats and oils in the oil industry, was chosen for selective extraction of squalene. Yield and selectivity of the extraction process was examined with the aid of response surface methodology (RSM). Chromatographic methods were applied to monitor squalene content and to characterize composition of the lipid fraction and optical microscopy to highlight changes in the degree of cell lysis among treatments.

MATERIALS AND METHODS

Lees Samples. All of the samples were obtained from the E. Tsantalis S. A. (Agios Pavlos, Greece). In 2010, samples were collected from the residue generated after the second decanting of wine ("light" lees) from seven white grape varieties (Chardonnay, Malagousia, Roditis, Sauvignon Blanc, Moschato Alexandrias, Opsimo Edessis, Moschofilero) and from two red ones (Xynomavro, Limnio) using

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different enological yeast strains (Table 1). Selective sampling of "light lees" (Γ34, Γ42) together with the respective streams of "heavy lees"

Table 1. Coded Numbers, Grape Varieties and Yeast Strains Corresponding to "Light Lees" Sampling in 2010

sample no	grape variety	yeast strain
white wines		
Γ28	Chardonnay	Vitilevure
Γ32	Malagousia	Zymaflore
Γ33	Roditis	Fermivin
Γ34	Chardonnay	Zymaflore X16
Γ35	Chardonnay	Enartis ez ferm
Γ42	Sauvignon Blanc	Zymaflore VL3
Δ27	Moschato Alexandrias	Zymaflore X5
E22	Opsimo Edessis	Fermivin
Z14	Moschofilero	Fermol Chardonnay
red wines		
B41	Xynomavro	Enartis vintage red
B42	Xynomavro	Fermirouge
I28	Limnio	Fermirouge

were collected in 2011. Moreover, "industrial waste", a mixture of wine lees generated from all of the wines produced in the same plant in 2011 period was included in the experimental design. Upon delivery to laboratory, lees sediments were separated by centrifugation of the samples at 3500*g* for 10 min and then were freeze-dried up to $a_w = 0.4$, to speed up the extraction process,[23] and stored at 4 °C until use.

Standards, Reagents and Solvents. Squalene (for biochemistry, purity 98%), was from Sigma Chemical Co. (St. Louis, MO, USA), ergosterol (purity >98%) from BDH Chemicals Ltd.. Poole, U.K.), DL-α-monoolein (38−40% diacyloglycerols, 16−18% triacyloglycerols, and 4−6% glycerol) from Fluka AG (Buchs, Switzerland). HPLC-grade solvents used were methanol, dichloromethane, acetone (Merck, Darmstadt, Germany), and acetonitrile (ChemLab, Ontario, CA, USA). Ultrahigh-purity water was delivered using a Millipore-Milli-Q-system (Barnstead International, Dubuque, IA, USA). All of the other common reagents and solvents were of the appropriate purity from various suppliers.

Apparatus. Water activity (a_w) of wine lees was measured using an AquaLab Lite water activity meter (Decagon Devices Inc., Pullman, WA, USA).

Experimental Design for the UAE. Wine lees were subjected to ultrasonication with standard probe (MS 72) (tip-diameter of 2 mm) in an ultrasonic processor (Ultrasonic Homogenizers HD 2070) for various time periods and cycles and at specific amplitude (97%). The sample temperature did not exceed 20 °C with the aid of an ice bath. The ultrasonic processor provided 70 W of power (at 20 kHz). An amount of the "industrial waste" (0.2 g) was added into the processing tube. Proper volume of *n*-hexane (20 mL) was added into the tube. The organic phase was separated by centrifugation at 4000*g* for 10 min. Solvent was removed under vacuum (40 °C) and the residue was weighted (±0.001 mg) and kept at −18 °C until further analysis.

Thirteen experiments were set according to an unblocked full factorial central composite statistical design[24] for the study of two factors, namely, sonication duration (min) (X_1) and duty cycles (active interval, s) (X_2), each at five experimental levels. The levels of the X_j factor were coded as follows: $-a$, −1, 0, +1, $+a$, where $a = 2^{n/4}$, n = number of variables, and −1, +1, and 0 correspond to the low, high and midlevel of X_j (Table 1 provided as Supporting Information). The thirteen runs were set using the software Minitab Release 13.20 (Minitab, Inc., State College. PA, USA) (Table 2). The design had five of the factorial points at the center of the design replicated for the estimation of error. Polynomial response surfaces, were fitted to the response variables, namely, squalene yield (SQY) (mg/kg lipid extract) and lipid yield (LY) (%, w/w dry lees). Statistical analysis of the experimental data was performed by RSM using the same software.

Table 2. Experimental Design for Five-Level-Two-Factor Central Composite Design and the Comparison between Observed and Predicted Responses for Squalene Yield (SQY) and Lipid Yield (LY) Obtained from "Industrial Waste"

	factors		actual/predicted	
	X1	X2		
run	duration of sonication (min)	duty cycles of sonication (active interval) (s)	SQY[a] (mg/kg of lipid extract)	LY[a] (%, w/w of dry lees)
1	15	0.6	14395 ± 987 /15344	1.50 ± 0.10 /1.54
2	5	0.9	7430 ± 341 /7992	1.55 ± 0.10 /1.62
3	29	0.6	19020 ± 457 /19650	2.00 ± 0.10 /1.93
4	5	0.3	5462 ± 500 /5538	1.15 ± 0.15 /1.19
5	15	0.6	15967 ± 987 /15344	1.60 ± 0.10 /1.54
6	15	0.6	14295 ± 987 /15344	1.50 ± 0.10 /1.54
7	1	0.6	4987 ± 208 /4505	1.20 ± 0.10 /1.14
8	15	0.2	10250 ± 583 /10668	1.40 ± 0.12 /1.36
9	15	0.6	16560 ± 987 /15344	1.50 ± 0.10 /1.54
10	25	0.3	17299 ± 479 /16589	1.45 ± 0.10 /1.50
11	15	1.0	13923 ± 787 /13654	2.40 ± 0.26 /2.31
12	25	0.9	18583 ± 967 /18359	2.34 ± 0.17 /2.42
13	15	0.6	15503 ± 987 /15344	1.60 ± 0.10 /1.54

[a]Mean value of three independent experiments ± SD.

Initially, the second-order polynomial model was fitted to each response giving an equation of the form:

$$\Upsilon = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

where Y is the predicted response, X_1 and X_2 represent the levels of the coded factors, and β_0, β_1, ..., β_{12} represent the estimated coefficients, β_0 being a scaling constant. The quality of the fit of the model was evaluated by the coefficients of determination (R^2), the significance of each parameter through F test, and the lack of fit of the model. Coefficients with a P value lower than 0.05 were considered significant. Where possible, the model was simplified by omission of nonstatistically significant terms. Optimization of the fitted polynomials for SQY and LY was performed using the software facilities. The combination of factor optimal values resulting in optimal response was verified experimentally in triplicate. Results were compared with model prediction outcomes.

Repeated extractions under optimum operational conditions (OOC) for SQY were performed by the following procedure: after sonication at duty cycle of 80% (active interval of 0.8 s) for 29 min the sediment was collected by centrifugation at 3500*g* for 10 min, and the extraction process was repeated with fresh solvent up to 4 times to recover the residual squalene.

Methods. *Preparation of the Unsaponifiable Matter (UM).* Squalene and sterols were extracted after methanolic pyrogallol saponification[10] with slight modification: an amount of freeze-dried lees (48 mg) was suspended in 5 mL 60% aqueous KOH solution, 7.5 mL methanol and 7.5 mL methanolic pyrogallol solution (0.5%, w/v) and incubated in a shaking incubator at 45 °C, overnight. The UM was extracted with hexane (3 × 10 mL). The phases were separated by centrifugation at 4000*g* for 10 min. The hexane layers were transferred

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to the same tube. Emulsions, if present, were dispersed by the addition of 0.5 mL methanol. The combined hexane fractions were dried over anhydrous Na_2SO_4 and finally the solvent was removed under vacuum (40 °C). Repeatability of extraction was satisfactory (CV% = 3.6 and 3.4, respectively, for squalene and ergosterol, n = 5). Crude extracts were kept at −18 °C until further analysis.

Total Lipids Extraction. Total lipids in lees were extracted using acid-assisted disruption of cells and determined as reported by Naziri et al.[12] Briefly, 0.1 g of freeze-dried lees sample was added to a sealed tube with 2.5 mL of hydrochloric acid solution (7.7 M) and 0.75 mL of water. The mixture was boiled for 10 min and, after cooling, carbon tetrachloride (2.5 mL) was added. The mixture was boiled again for 10 min, cooled, and then diethyl ether-ethyl petroleum (1:1, v/v) (18.75 mL) was added. After vigorous shaking (5 min), an aliquot of the upper phase (15 mL) was obtained. The solvent was removed under vacuum (40 °C) and the obtained lipids were weighted (±0.001 mg). The lipid concentration was calculated per 100 g of freeze-dried lees sample. Repeatability of extraction was satisfactory (CV% = 2.8, n = 5). Lipids were kept at −18 °C until further analysis.

Separation of Lipid Classes by Thin-Layer Chromatography (TLC). Separation was achieved on thin layer silica gel plates, 20 × 20 cm, 0.2 mm (Merck, Darmstadt, Germany). The separation of neutral lipid classes was accomplished in two steps by two solvent mixtures of different polarity (System I).[25] The first developing mixture of solvents was petroleum ether-diethyl ether-acetic acid (40:40:1.6, v/v/v) and it was developed up to the 1/3 of the plate. The second one consisted of petroleum ether/diethyl ether (78.4:1.6, v/v) and it was developed up to the remaining 2/3 of the plate. Spots were visualized using H_2SO_4 solution (50%, v/v) followed by heating for 10 min in an oven (130 °C). Phospholipid classes were separated in two steps on another TLC plate (System II).[26] The first solvent was acetone and it was developed 18 cm above the origin. The second mixture of solvents was chloroform−methanol−acetic acid−water (25:15:4:2, v/v/v/v) and it was developed up to the height of 17 cm. Visualization of spots was as mentioned above. Spots were identified by comparison with authentic standards (squalene, triacylglycerols, diacylglycerols, monoacylglycerols, ergosterol) or by literature retention factors (R_f)[26] or by fluorescence characteristics.[27]

RP-HPLC of Squalene and Ergosterol. The UM was dissolved in methanol-dichloromethane (9:1, v/v). All of the samples were filtered through a 0.45 μm membrane filter just before HPLC analysis. Separation of squalene and sterols was carried out on a reversed phase Nucleosil C_{18} column (250 × 4.6 mm i.d.; 5 μm) (Macherey-Nagel, Duren, Germany). Elution with methanol−water 98:2 (v/v) was assisted by maintaining column temperature at 40 °C. The flow rate was set at 1.0 mL/min and the injection volume was 10 μL. The solvent delivery system consisted of two Marathon IV Series HPLC pumps (Rigas Laboratories, Thessaloniki, Greece), a Rheodyne 7125 injection valve with a 20 μL fixed loop (Rheodyne 7125 LP, Cotati, CA, USA). The chromatograph was coupled to a Linear UVVIS-206 diode array multiple-wavelength detector (Linear Instruments, Fremont, CA, USA). Chromatographic data were processed with the software EZChrom 6.6 (Sci Software, Inc., San Ramon, CA, USA). Detection and quantification of squalene and ergosterol was at 208 and 285 nm, respectively. Peak identification was achieved by comparison of the retention time with that of authentic standards, and confirmed by spiking and comparison of spectral data. Quantification was accomplished with the aid of a standard curve calculated by linear regression analysis. Analysis of samples was carried out in duplicate (CV% = 2.3 and 2.5, respectively, for a 50 mg/L squalene and a 500 mg/L ergosterol standard solution, n = 5). Squalene content in the lipid extract was determined on a reversed phase LiChroCART column (125 × 4.0 mm i.d.; 4 μm) maintained at 26 °C. The elution solvent was 100% acetonitrile, the flow rate 1.2 mL/min, and the injection volume 10 μL. The HPLC system consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA, USA), a Midas autosampler (Spark, Emmen, The Netherlands), and a UV 6000 LP diode array detector (DAD; Thermo Separation Products). Detection and quantification of squalene was at 208 nm. Analysis of samples was carried out in duplicate (CV% = 2.1 for a 50 mg/L squalene, n = 5). The column was carefully washed out using acetone after squalene elution.

RP-HPLC of Triacylglycerols. The lipid extract was dissolved in acetone (10%, w/v) and was filtered through a 0.45 μm membrane filter just before HPLC analysis. The elution solvent was acetone−acetonitrile 60:40 (v/v). Separation of triacyloglycerols (TAGs) was achieved isocratically on a Nucleosil C18 column (250 × 4 mm i.d; 5 μm.) at a flow rate of 1.2 mL/min. The injection volume was 10 μL. The solvent delivery system consisted of an SSI liquid chromatography pump (model 300; Scientific Systems Inc., State College, PA. USA). Separation of triacylglycerols was performed using refractive index detection (RID-6A, Shimadzu Co., Tokyo, Japan). Characterization of TAG groups according to equivalent carbon number (ECN) was based on retention times of those of a virgin olive oil.

GC−MS of Fatty Acid Methyl (FAMEs) and Ethyl (FAEEs) Esters. Fatty acid methyl esters were prepared using the alkali trans-esterification procedure proposed to the Commission Regulation (EEC, No. 2568/91),[28] after slight modifications. The FAMEs were analyzed along with endogenous FAEEs, on an Agilent 6890A gas chromatograph (GC) equipped with MSD 5973 mass spectrometer (MS) (Palo Alto, CA, USA). Separation of compounds was achieved on a FFAP fused capillary column (25 m × 200 μm × 0.33 μm) (Agilent J&W, Palo Alto, CA, USA) under the following standard operation conditions: carrier gas, helium (1 mL/min); oven temperature gradient: 35 (5 min); 35−200 at 15 °C/min, 200 °C (15 min), 200−230 at 15 °C/min, 230 °C (5 min); transfer line temperature, 230 °C. MS was taken at 70 eV with a scan range between 35 and 350 amu at 2 scans/s. Compounds were identified by use of NIST library (Version 2.0d, 2005).

GC−FID of Fatty Acid Methyl (FAMEs) and Ethyl (FAEEs) Esters. Fatty acid methyl and ethyl esters were also analyzed on an Agilent 6890 gas chromatograph (GC) coupled with a flame ionization detector (FID). Separation of compounds was achieved on an FFAP fused capillary column (25 m × 200 μm × 0.33 μm) under the same GC−MS operation conditions. Injector and detector temperatures were kept at 230 °C. The percent of the individual FAMEs and FAEEs was calculated on the basis of the total area of the peaks present.

Optical Microscopy. A representative amount of treated wine lees samples was suspended in deionized sterile water and 0.1 mL suspension was mounted between a glass slide and a coverslip and examined using a Zeiss Axiolab reflected light microscope (100× magnitude) equipped with a Canon Power Shot G 2 mm (Canon, Tokyo, Japan) photographic camera. For each treatment, three independent samples were examined under the microscope.

RESULTS AND DISCUSSION

Squalene Accumulation in Lees from White and Red Wines. At a first stage, differences in the squalene content of lees obtained from various grape varieties (harvest 2010) and yeast strains within the white or red vinification process were sought. Special emphasis was given to the "light" lees, the richest winery waste in *S. cerevisiae* cells.[2,3]

In all cases, HPLC of the UM of lipids pointed out the selective accumulation of squalene (Figure 1, peak 1). Quantitative determination of squalene in the UM of the samples (Table 3) highlighted differences due to genetic or technological effects. Specifically, the levels of squalene in white wine lees ranged from 2.43 to 5.90 (mg/g dry lees), whereas lower values were found for red wine lees (0.54−1.54 mg/g dry lees). In white winemaking, excessive contact with oxygen is avoided to retard the browning reactions and protect overall quality, whereas in red winemaking, the maceration step that enhances extraction of phenolic compounds and pigments from the grape skins favors oxygenation of the must. The above results are consistent with those of a previous study on the positive effect of limited oxygen supply to squalene accumulation in *S. cerevisiae* cells.[10] In addition, the indirect

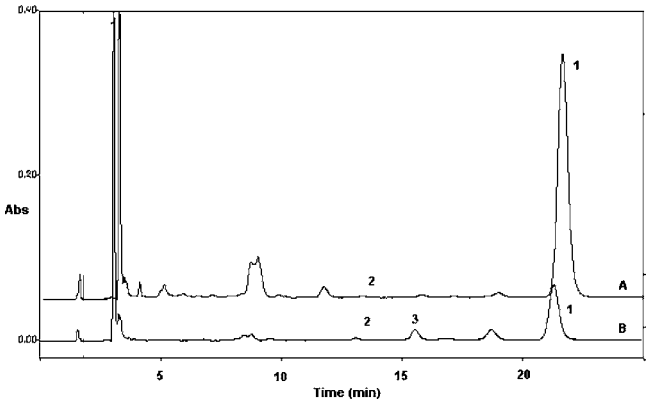

Figure 1. RP-HPLC of squalene and ergosterol in the UM of lipids extracted from "light" lees generated from Γ42 (A) and B42 wines (B). Peaks: 1, squalene; 2, ergosterol; 3, 22,23-dihydroergosterol. Chromatographic conditions as under Materials and Methods.

Table 3. Squalene (SQC) and Ergosterol (ERGC) Content of Lees Generated from Different Types of Wines

sample no.	SQC[a] (mg/g dry lees)	ERGC[a] (mg/g dry lees)
white wines		
Γ28	4.53 ± 0.19	0.06 ± 0.014
Γ32	3.70 ± 0.28	0.08 ± 0.024
Γ33	3.19 ± 0.25	0.06 ± 0.021
Γ34	5.64 ± 0.42	0.22 ± 0.022
Γ35	2.43 ± 0.19	0.06 ± 0.006
Γ42	5.90 ± 0.43	0.34 ± 0.016
Δ27	2.61 ± 0.27	0.04 ± 0.014
E22	3.46 ± 0.34	0.11 ± 0.050
Z14	3.79 ± 0.27	0.25 ± 0.014
red wines		
B41	0.54 ± 0.04	0.16 ± 0.04
B42	1.54 ± 0.10	0.31 ± 0.02
I28	1.29 ± 0.09	0.21 ± 0.02

[a]Mean value of three independent experiments ± SD.

effect of ethanol produced should not be precluded, as many of the important genes (*ERG25*, *ERG2*, *ERG3*) that act downstream in squalene synthesis are down-regulated under ethanol stress.[29] Among lees from Chardonnay wine, variations were observed only when different strains were used. These results indicate that the squalene content in wine lees is under the control of yeast strain genetic factors. Since acetoacetyl-CoA thiolase and HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) synthase (the first two enzymes of the presqualene pathway) are known to be induced by ergosterol starvation conditions,[30] it is expected that higher squalene content in lees is the result of a more active genetic regulation of its synthesis. Genetic variation was also a key factor in the case of red wine lees (samples B41 and B42) despite the intervention of technology. Grape variety itself was found to influence less squalene accumulation in both vinification processes. In practice, all of the above variations are expected to even out as the "industrial waste" lees are a mixture of waste from different winemaking processes.

Spectrum of sterols present in the extracts was restricted to a few ones in low amounts. Ergosterol content that was detected in the lees ranged from 0.04 to 0.34 mg/g dry lees as shown in Table 3. In the red-wine vinification process, the effect of oxygen should normally be accompanied by a concomitant increase in the sterol content. However, data did not indicate higher ergosterol content in red lees compared with that in white ones. Trying to avoid speculation, findings may be attributed to (a) the effect of excessive levels of oxygen during fermentation that resulted in the oxidation of ergosterol and accumulation of oxidized sterols (e.g., 22,23-dihydroergosterol) (Figure 1B, peak 3)[9,10] and/or (b) the substitution of ergosterol by grape phytosterols in the yeast membrane promoting growth and initial fermentative activity.[31]

Selective sampling of "light" lees was repeated in 2011. Selection was based on the yield observed the previous year. In addition, the respective streams of "heavy" lees also collected. In all cases, squalene yields from "light" lees were similar to the respective ones from the previous harvest. As expected,[2] "heavy" lees were found to contain lower squalene content than those obtained from "light" lees (5.05 vs 0.73 and 4.53 vs 0.82 mg/g dry lees, respectively). Nevertheless, both categories are of industrial interest since "heavy" lees stream represents large volumes of waste (5%, v/v). Findings so far supported the aim of the present study, which was the valorization of industrial wine lees waste, i.e., mixture of lees from the different winemaking processes. Thus, in the optimization studies, the waste stream used was the mixture of wine lees generated from all vinification processes of the particular industry in 2011.

Optimization of the UAE Parameters for the Recovery of a Lipid Fraction Enriched in Squalene Using the RSM. A central composite design (CCD) was applied to select ultrasound experimental conditions. The potential influence of the two independent factors, namely duration (X_1) and duty cycles (X_2) of sonication, to the squalene yield (SQY) (mg/kg lipid extract) and the lipid yield (LY) (%, w/w dry lees) from mixture of lees from the different winemaking processes was examined. The statistical model fitted to the data for the responses allowed assessment of interactions among these factors through a reduced number of experiments. The full second-order models (eqs 1−4), in terms of coded (−1, +1) and actual factor levels (Table 1 provided as Supporting Information), fitted to the data from the CCD for each one of the response variables are shown in Table 2 (provided as Supporting Information). Statistical details and description of model fitting are provided as Supporting Information (text and data in Tables 3 and 4).

The fitted polynomial equations (eqs 2 and 4 in Table 2 provided as Supporting Information) were expressed as response surface plots to visualize relationships between SQY and LY and factors (X_1, X_2) and to deduce the OOC (Figure 2A,B). It is well documented that ultrasonic waves create cavitation phenomena causing cell lysis, thus facilitating the release of intracellular material (i.e., lipids) into the solvent. In addition, the mechanical effect of ultrasound enhances mass transfer, allowing a better penetration of solvent into the cells.[20] As shown in Figure 2, UAE under mild operational conditions (i.e., low duty cycles and short treatment duration) resulted in poor recoveries of squalene and total lipids. It seems that hexane, having a high vapor pressure value (132 vs 33 and 17.3 mmHg at 20 °C, for isopropanol and water, respectively), inversely affected cavitation intensity.[19] Higher duty cycles and longer treatment duration gave rise to the damage of the cell walls facilitating the release of higher amounts of squalene and other lipids, as evidenced under optical microscopy observation (Figure Ia,b provided as Supporting Information). In Figure 2A, the characteristic curvature of the response surface illustrates the negative quadratic effects of the above factors to SQY

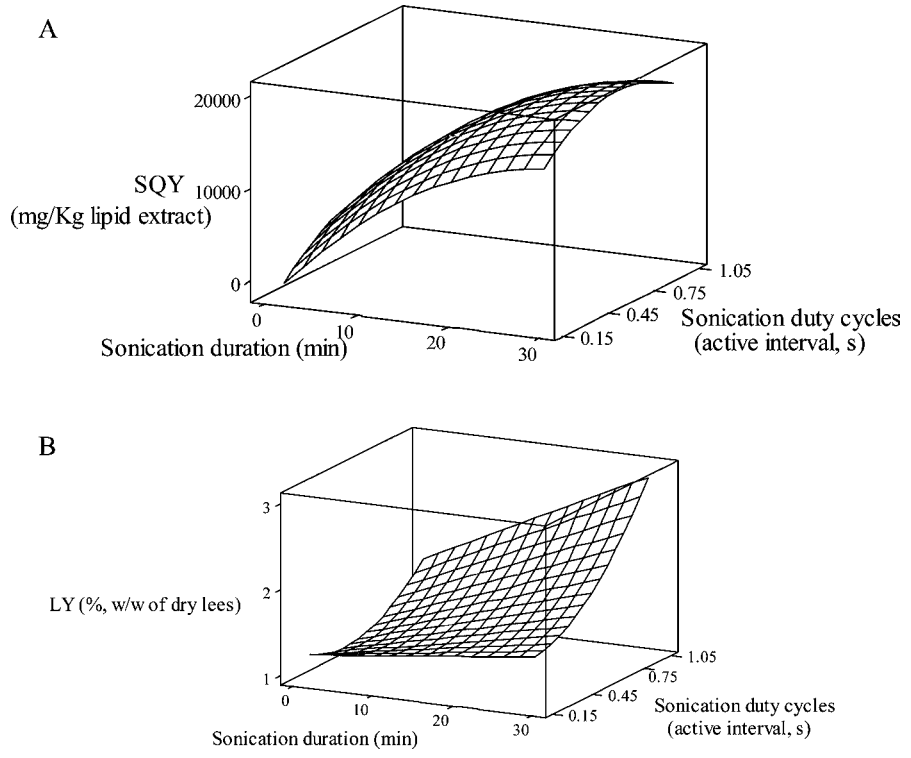

Figure 2. Surface plot for "industrial waste" (A) squalene yield (SQY) and (B) lipid yield (LY) at varying levels of sonication duration (min) and duty cycles (active interval) (s).

indicating that this response is not favored by the continuous operation of ultrasound (pulsation rate 1 s active) for long treatment duration. This observation raised concern for partial decomposition of squalene under the above operational conditions, although the increased duration and duty cycles of sonication resulted in a higher LY (Figure 2B).

Predicted and Verified Optimum Conditions for SQY and LY. As the fitted models for eqs 2 and 4 (Table 2 provided as Supporting Information) provide a good approximation to the experimental conditions, these were employed to predict optimum values of the process variables for the maximum SQY and LY. The predicted ones for optimum SQY were sonication at duty cycle of 80% (active interval of 0.8 s) for 29 min. For LY, the respective values were continuous operation of sonication (active interval of 1 s) for 29 min. Then, the above sets of conditions were substituted to eqs 2 and 4, respectively, to obtain maximum SQY and LY responses, which were 21 090 (mg/kg lipid extract) and 2.90 (%, w/w dry lees), respectively (Table 4). The experimental values of responses, shown in the same table, fit well with the corresponding predicted ones. In particular, maximum SQY and LY from mixture of lees from the different winemaking processes obtained were 20 390 ± 1335 mg/kg lipid extract (0.45 g/kg dry lees) and 2.50 ± 0.21 (%, w/w dry lees), respectively.

Under the OOC for LY, lipid recovery accounted for about 60% of the value obtained by acid treatment of lees prior to extraction (2.50 ± 0.21 vs 4.24 ± 0.34%, w/w dry lees, respectively). The latter method is considered as the most effective one for yeast autolysis[23] (Figure Ic provided as Supporting Information). Regarding SQY, OOC was more selective than acid treatment (20390 ± 1335 vs 9102 ± 1192 mg SQ/kg lipid extract, respectively). This was verified by exhaustive extractions under the OOC, up to 4 times, which provided an additional lipid yield of 1.73 ± 0.16 (%, w/w dry lees) and a squalene content of 8898 ± 1152 mg SQ/kg lipid extract (0.16 g/kg dry lees). The selectivity of the OOC was also confirmed in comparison to that found in the hexane extract of lees lysed by liquid nitrogen grinding (20390 ± 1335 vs 8954 ± 895 mg SQ/kg lipid extract). Moreover, in comparison to recently examined potential sources, this value is comparable (0.2−0.35 g SQ/kg dry olive pomace and 0.06 g SQ/kg olive leaves).[32,33]

Table 4. Optimum Values of Sonication Duration and Duty Cycles (Active Interval) and Maximum Predicted and Experimental Values of Squalene Yield (SQY) and Lipid Yield (LY) Obtained from "Industrial Waste"

		SQY (mg/kg of lipid extract)	
factor	optimum actual values	predicted response value	mean experimental response value[a]
duration of sonication (min)	29		
duty cycles of sonication (active interval) (s)	0.8	21090	20390 ± 1335
		LY (%, w/w of dry lees)	
		predicted response value	mean experimental response value[a]
duration of sonication (min)	29	2.90	
duty cycles of sonication (active interval) (s)	1.0		2.50 ± 0.21

[a]Mean value of three independent experiments ± SD.

Characterization of the Lipid Fraction Enriched in Squalene. Examination of lipid composition by TLC system I verified the presence of squalene (R_f = 0.89), squalene oxidation products (R_f = 0.81), steryl esters (R_f = 0.57−0.48), fatty acid ethyl and methyl esters (FAEEs, FAMEs) (R_f = 0.42),

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triacylglycerols (R_f = 0.22), diacylglycerols (R_f = 0.17), free sterols (R_f = 0.14), and pigments (R_f 0.11−0.06). The deep red fluorescence under long wave UV light of the pigments along with data from the visible spectrum of this lipid fraction (410, 470, 506, 536, 608, 667 nm) suggested the presence of chlorophyll-derived compounds (R_f = 0.11, 0.08, 0.06).[27,34] Phospholipids (TLC system II) were not detected in the lipid extract. Gomez et al.,[18] the only researchers who gave information concerning the lipid composition of wine lees based on HPTLC results, reported the presence of steryl esters, fatty acid methyl esters, triacylglycerols, free fatty acids, free sterols, glycolipids, and phospholipids, but not the presence of squalene. Differences in lipid classes evidenced among researchers may be partially due to means of extraction and solvent polarity. In the present study, the presence of squalene is justified by the UAE with hexane, whereas its absence in the paper by Gomez and co-workers[18] should be related to milder extraction means (stirring, 30 min) using *n*-butanol saturated with water. Steryl esters, attributed to yeast cell composition, along with triacylglycerols comprise the majority of lipids of lees according to Pérez-Serradilla et al.[3] Fatty acid ethyl esters and methyl esters, products of esterification of free fatty acids with residual ethanol and methanol, are expected under the same spot as verified on additional TLC runs. Free fatty acids were not detected, though reported in literature. Phospholipids were not detected in the lipid fraction even when extraction solvent (hexane) was replaced by petroleum ether-diethyl ether-carbon tetrachloride (0.45:0.45:0.1, v/v/v) mixture. Absence of phospholipids may be attributed to degradation after cell lysis.[3,16] All of the above lipid classes are justified by the composition of either yeast or grapes.[23,31,34−37]

Fatty acid methyl ester composition of the lipid fraction by GC−MS and GC−FID analyses identified the presence of stearic, palmitic, myristic, lauric, capric, oleic, linoleic, and linolenic acids. The presence of certain fatty acids (e.g., margaric, erucic) reported by Gomez et al.,[18] can neither be justified by the composition of *S. cereviciae* cell lipids nor by that of grapes and should be the outcome of misidentification of chromatographic peaks. The major triacyloglycerols species separated by RP−HPLC coupled to a refractive index detector was that of ECN 46. The peaks noticed in the beginning of the chromatogram may indicate the presence of diacylglycerols derived from yeast cells.[23]

The obtained lipid fraction was found to be more complex than the one extracted from different commercial yeast strains.[12] Considering that squalene content accounted for only the 2% (w/w) of this fraction, the presence of other components may affect its stability. Squalene loss in lipid fractions obtained by different methods can be high as indicated in preliminary stability experiments (Figure II provided as Supporting Information). This observation needs a thorough investigation, which was beyond the aim of the present feasibility study.

Wine lees can be considered as a potential source for squalene, having pros and cons. Among the pros are the raw material availability and the perspective of a cost-effective, environmentally friendly investment by the winery industry. The latter is expected to create an additional source of input with a concomitant decrease in disposal expense. The economic benefit of the proposed valorization route is expected to increase if recovery of more than one target compound from lees is designed (e.g., tartaric acid and nutrient for *Lactobacillus*). The cons include (a) the seasonal character of this waste, (b) its handling requirement, and (c) a complex lipid fraction that may affect squalene stability.

■ ASSOCIATED CONTENT

Supporting Information

Table 1. Levels of factors in actual and coded values used in the experimental design. Table 2. Model equations for prediction of the optimum response values of "industrial waste" SQY and LY. Table 3. Analysis of variance of "industrial waste" SQY and LY obtained using the RSM model. Table 4. Estimated regression coefficients and significance (P values) for "industrial waste" SQY and LY after analysis using coded values of factors. Figure I. Microphotographs of wine lees (solid fraction) without (a), and after ultrasound (active interval of 0.9 s, 25 min) (b) or acid assisted (c) treatment. Figure II. Chromatographic profile of the lipid extract derived under OOC − gray: 0 days, black: 5 days (a), acid assisted extraction − gray: 0 days, black: 18 days (b); mild conditions (stirring 18 h, lees lysed by liquid nitrogen grinding) − gray: 0 days, black: 5 days (c). Peak 1: squalene (chromatographic conditions as in Materials and Methods Section). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

UAE, ultrasound assisted extraction; RSM, response surface methodology; SQY, squalene yield; LY, lipid yield; OOC, optimum operational conditions; UM, unsaponifiable matter; TLC, thin-layer chromatography; FAMEs, fatty acid methyl esters; FAEEs, fatty acid ethyl esters; CCD, central composite design.

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