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Rapid Quantification of Yeast Lipid using Microwave-Assisted Total Lipid Extraction and HPLC-CAD

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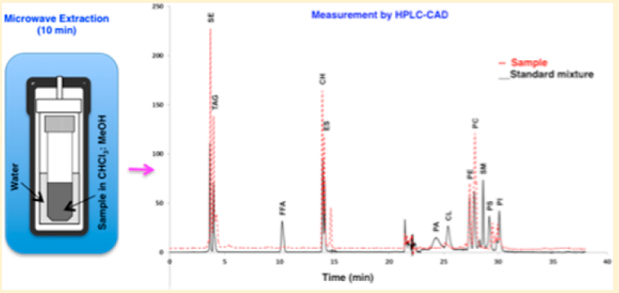
Supporting Information

ABSTRACT: We here present simple and rapid methods for fast screening of yeast lipids in *Saccharomyces cerevisiae*. First we introduced a microwave-assisted technique for fast lipid extraction that allows the extraction of lipids within 10 min. The new method enhances extraction rate by 27 times, while maintaining product yields comparable to conventional methods (n = 14, P > 0.05). The recovery (n = 3) from spiking of synthetic standards were 92 ± 6% for cholesterol, 95 ± 4% for triacylglycerol, and 92 ± 4% for free fatty acids. Additionally, the new extraction method combines cell disruption and extraction in one step, and the approach, therefore, not only greatly simplifies sample handling but also reduces analysis time and minimizes sample loss during sample preparation. Second, we developed a chromatographic separation that allowed separation of neutral and polar lipids from the extracted samples within a single run. The separation was performed based on a three gradient solvent system combined with hydrophilic interaction liquid chromatography-HPLC followed by detection using a charged aerosol detector. The method was shown to be highly reproducible in terms of retention time of the analytes (intraday; 0.002−0.034% RSD; n = 10, interday; 0.04−1.35% RSD; n = 5) and peak area (intraday; 0.63−6% RSD; n = 10, interday; 4−12% RSD; n = 5).



Lipids are energy storage molecules and important structural components of all eukaryotic cell membranes.[1] Yeast cell membranes are composed of three main components: phospholipids, sterols, and intramembrane proteins.[2−4] The principal sterol in *Saccharomyces cerevisiae* is ergosterol.[4] The principle phospholipids in this organism have been shown to be cardiolipin (CL), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) with fatty acid (FA) chains that are predominantly oleic acid and palmitoleic acid, with smaller amounts of palmitic acid and stearic acid, and very low amounts of myristic acid.[5−7] Like other eukaryotes, yeast cells also have a pool of neutral lipids stored as cytoplasmic droplets (which serve as reservoirs of cellular energy and building blocks for membrane lipids) consisting of triacylglycerols (TAG) and steryl esters (SE)[8,9] surrounded by a monolayer of phospholipids and associated proteins.[2,10,11] In yeast, the lipid droplets consist of TAG and SE in a ratio of about 1:1.[11−13] The total amount of lipids stored in lipid droplets is in general considered to be low relative to the dry cell mass (<15%), but the amount of neutral lipid storage in yeast is probably highly dynamic. Yeasts, as unicellular organisms, are able to quickly and easily adjust their internal metabolism to new conditions. Indeed, environmental stress and starvation have been shown to induce increased synthesis and accumulation of neutral lipids.[11,14,15]

Lipid extraction is the first step in lipid analysis, lipids from tissue or microorganisms are normally extracted by partitioning into the organic solvents, mostly with the mixture of chloroform−methanol[16,17] or by methyl *t*-butyl ether.[18] For yeast, lipids are most efficiently extracted from freeze-dried or freeze-thawed cells.[19] In addition, mechanical disintegration of the cells (sonication and bead mills), cell wall digestion (using zymolyase), or drying yeast at moderate temperatures can enhance the efficiency of lipid extraction.[4,20−22] The addition of these steps for sample preparation and extraction do, however, increase labor time but also requires skills to perform each specific step and increases the chance of errors because of sample loss during these multiple steps.

Recently, microwave technology has been introduced for fast sample preparation for lipid analysis, mostly for performing fatty acid methyl esters (FAMEs) analysis in several eukaryotic cells, such as plant, animal, and fungal cells.[23−28] To improve the sampling time, we recently developed a modified closed-vessel method with microwave-assisted extraction.[29] With the

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new approach, we are able to reduce the time for preparation of FAMEs from 120 to 5 min and hereby could increase the sample preparation rate to several hundred samples per day. The combination of microwave technology for total lipid extraction with analysis of lipids has, however, not been studied before.

Several chromatographic methods have been developed for separation and quantification of plant, animal, and microbial lipids.[30,31] Among these, hydrophilic interaction liquid chromatography (HILIC) columns have been recently introduced for metabolomic profiling and lipid profiling.[32] This type of column is based on normal phase chromatography, where a polar stationary phase is used together with an associated layer of water that promotes chromatographic separation.[33,34]

Although mass spectrometers are extremely powerful for identification and quantification of lipids,[5] these techniques are associated with high operation costs and require skilled operators. The most simple and inexpensive detection for HPLC-based lipid analysis is ultraviolet (UV) detection; however, conventional UV detection is often not adequate and it is limited to chromophores.[35] On the other hand, other detection methods, such as flame ionization detection (FID) or evaporative light-scattering detection (ELSD), have significant limitations in precision, sensitivity, and dynamic range.[36−38] Recently a new type of universal detector, the so-called charged aerosol detector (CAD), has been developed[37,39] and introduced for analysis of lipids. Previous work[37,40] showed that lipid classes can be separated and quantified using a normal phase column in combination with a CAD detector. However, the necessity to perform two runs to complete the separation of polar and nonpolar lipids greatly complicates the analysis and results in a long run time. Although several HPLC-based methods can separate polar and nonpolar-lipid classes in a single run, none of those reported so far has used CAD detector.

Here, we present a new method for rapid extraction of yeast lipids using microwave technology, followed by HPLC-CAD for the low resolution of lipid class analysis. The methods were validated and demonstrated for use in analysis of yeast lipids and potentially useful for other organisms.

MATERIAL AND METHODS

Chemicals and Standards. All solvents and reagents and lipid standards used in this study were analytical grade, purchased from Sigma-Aldrich, Germany.

Yeast Strain and Cultivation Conditions. The yeast strain CEN.PK 113-7D MATα SUC2MAL2-8C (Scientific Research and Development GmbH, Germany) were grown aerobically in 50 mL YPD in 500-mL baffled shake flasks at 30 °C and 230 rpm. The initial glucose was 20 g/L concentration and the initial cell concentrations corresponding to an OD_{600} 0.01 were inoculated.

Samples were harvested from the cultivation media during the stationary phase at 36 h, transferred into 50 mL-falcon tubes (VWR, Sweden) and centrifuged at a speed of 3000 rpm (1912g) for 5 min at 4 °C to collect the biomass. The samples were then immediately frozen in liquid nitrogen and placed in freeze-dryer at −40 °C overnight.

Lipid Extraction Recipes. *Conventional Lipid Extraction.* Before the extraction, the freeze-dried yeast cells were disrupted by mechanical cell breaking using the method described in our previous study.[29] Briefly, 0.5 mL of digestion buffer was added to yeast cells (∼10 mg), followed by 50 μg of cholesterol (CH) in Pyrex tube. Thereafter, 20−30 acid washed glass bead (425−600 μm) were added and vigorously vortexed for 45 min. The tube was then placed in freeze-drier (alpha 2−4 LSC, CHRIST, Gmbh, Germany) to remove digestion buffer at −40 °C at 1.03 atm overnight.

After the cell disruption process, the lipids from yeast cells were extracted according to the conventional method.[22] Briefly, 7 mL of chloroform−methanol (2:1, v/v) was added into the tube, flushed with N_2 gas (30s) and closed tightly with a Teflon screw cap. The tube was vortexed at 300 rpm at room temperature for 3 h, allowing for an extraction process. After 3 h, 1.7 mL of NaCl (0.73% w/v) was added into the tube and centrifuged at 3000 rpm (1912g) at 4 °C for 10 min allowing for phase separation. The organic-phase (lower phase) was collected and the remaining phase was re-extracted with 5 mL of chloroform−methanol (85:15 v/v) for another 1.5 h. The organic phase from the second extraction was collected and pooled with the previous organic fraction. The extracted sample was then concentrated by drying under vacuum, resuspended with 200 μL of chloroform−methanol (2:1 v/v) and analyzed by HPLC-CAD.

Microwave-Assisted Lipid Extraction. The experimental set up for microwave extraction was slightly modified from our previous work.[29] Freeze-dried cells (∼10 mg) were mixed with the internal standard (50 μg of CH) and 7 mL of chloroform−methanol (2:1, v/v) in the extraction tube. After flushing the tube with N_2 gas (30s), it was vigorously vortexed before placing in the microwave reaction vessel (12 cm ×3 cm I.D., 0.5 cm thickness; Milestone Stard D, Sorisole Bergamo, Italy) that contained 30 mL of Mili-Q water inside and then sealed with a TFM screw cap. The vessel was heated using a microwave digestion system equipped with PRO-24 medium-pressure high-throughput rotor (Milestone Stard D, Sorisole Bergamo, Italy).

The temperature programing of microwave extraction was ramped to 60 °C (from room temperature, using 800 W for 24 vessels) within 6 min and kept constant for 10 min. After the sample was cooled down to room temperature, 1.7 mL of NaCl (0.73% w/v) was added, and then the sample was vortexed vigorously. Thereafter, the sample was centrifuged at 3000 rpm (1912g) for 10 min allowing for phase separation and the organic phase was transferred into a new clean extraction tube. The extracted sample was then preconcentrated by drying under vacuum, resuspended with 200 μL of chloroform−methanol (2:1, v/v) and further analyzed by HPLC-CAD.

Lipid Analysis via HPLC-CAD. Lipid separation and quantification were developed based on the method from Silversand and Haux.[41] Lipid separation was accomplished by HPLC (Dionex; ultimate 3000 HPLC system, Germany) equipped with a CAD detector (Corona; ESA, Chelmsford, MA, U.S.A.) supplied with N_2 at 35 psi gas pressure. The chromatogram was recorded at 10 Hz frequency and gain at 100 pA. A 2 μL volume of sample (from the Lipid Extraction Recipes section) was injected into the Luna 5 μm HILIC 200 A, 250 × 4.6 mm (normal phase) from Phenomenex, at 35 °C with the flow rate of 0.8 mL/min. The mobile phase was comprised of three different solvent systems as followed: (A) hexane−acetic acid (99:1, v/v), (B) acetone−isopropanol−acetic acid (29:70:1, v/v), (C) water−acetone−isopropanol−acetic acid (9:20:70:1, v/v) and triethylamine (0.08%, v/v) was added to adjust pH to reach 5.0. The gradient elution started with 100% of solvent A (at 0 min) and its fraction varied

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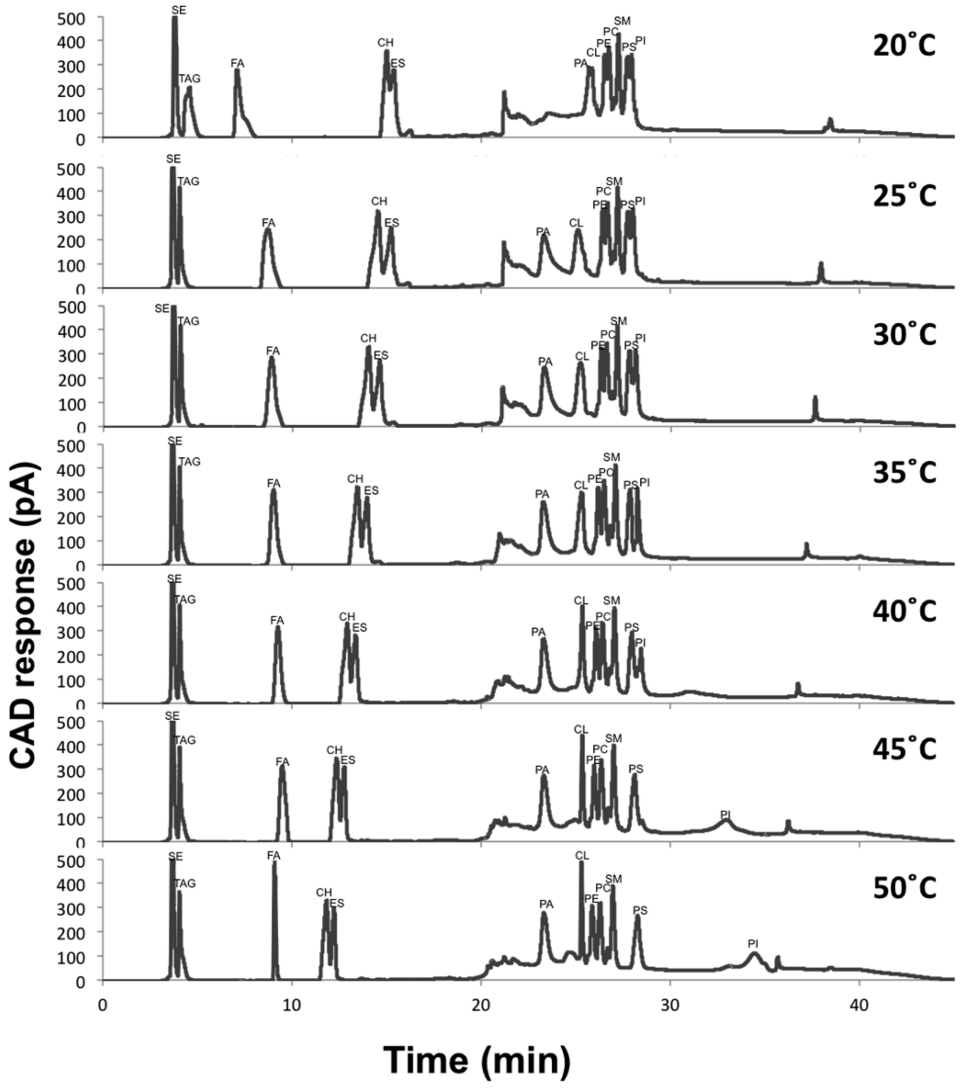

Figure 1. Effect of column temperature on the separation of all lipid classes, separated on a HILIC column (Luna 5 μm 200 Å 250 × 4.6 mm. 0.8 mL/min solvent flow rate) with triple gradient mobile phase.

depending on the solvent B and C during the entire process of 45.9 min. The solvent B was gradually added to the system to reach 1% (at 5 min), 2% (at 6 min), 3% (at 14 min), 5% (at 19 min; maintained until 36 min), 20% (at 38 min), 2% (at 40 min), and finally 0% (at 42 min). The solvent C gradient reached 0.5% (at 14 min), 35%(at 19 min), 44% (at 36 min) and finally 0% (at 38 min). Identification of unknown lipids extracted from yeast was performed in two ways. First, using HPLC-CAD, we compared the retention times of unknown lipids with known standards under the identical chromatographic conditions. Second, we connected a fraction collector to our HPLC-CAD system and fractionated lipids for further characterization using mass spectrometry (MS). The lipid classes were confirmed using lipid class-specific scans on a QTRAP 5500 mass spectrometer (ABSciex, Toronto, Canada) equipped with a robotic nanoflow ion source NanomateTriversa (Advion Biosciences, Ithaca, NY, U.S.A.). See supplement for further information.

The quantification of lipids was performed using external calibration curves from known lipid standards (SE, TAG, FA, CH, ES, PA, CL, PE, PC, SM, PS, and PI) within the range concentrations of 10−1000 μg/mL (2 μL injected). Each concentration of the standard solutions was injected twice and the average $\log_{10}$ of peak area was plotted against $\log_{10}$ of the concentration. Correlation (r^2) was determined for all standard curves by linear regression.

FAME Analysis. The parameters used for the measurement of FAMEs in this study were set according to our previous study.[29] Briefly, the collected fractions from fraction collector were mixed with 4 mL of hexane, 2 mL of 14% BF_3 (in MeOH) in an extraction tube (Pyrex borosilicate glass 16 × 100 mm, U.S.A.). The solution was then flushed with nitrogen gas for 30 s and closed tightly with a Teflon screw cap. The tube was placed in a vessel (12 cm × 3 I.D., 0.5 cm thickness; Milestone Start D, Sorisole Bergamo, Italy) containing 30 mL of Milli-Q water and then sealed with a TFM screw cap. The vessel was then heated using a microwave instrument (Milestone Start D, Sorisole Bergamo, Italy). The temperature program was ramped to 120 °C (500 W for 4 vessels) within 6 min and maintained for 5 min. The upper phase (hexane) containing FAMEs was analyzed by GC-MS. The GC-MS measurements were performed in a splitless mode (1 μL at 240 °C) and helium was used as a carrier gas (1 mL/min). The column temperature was initially set at 50 °C (1.5 min) and

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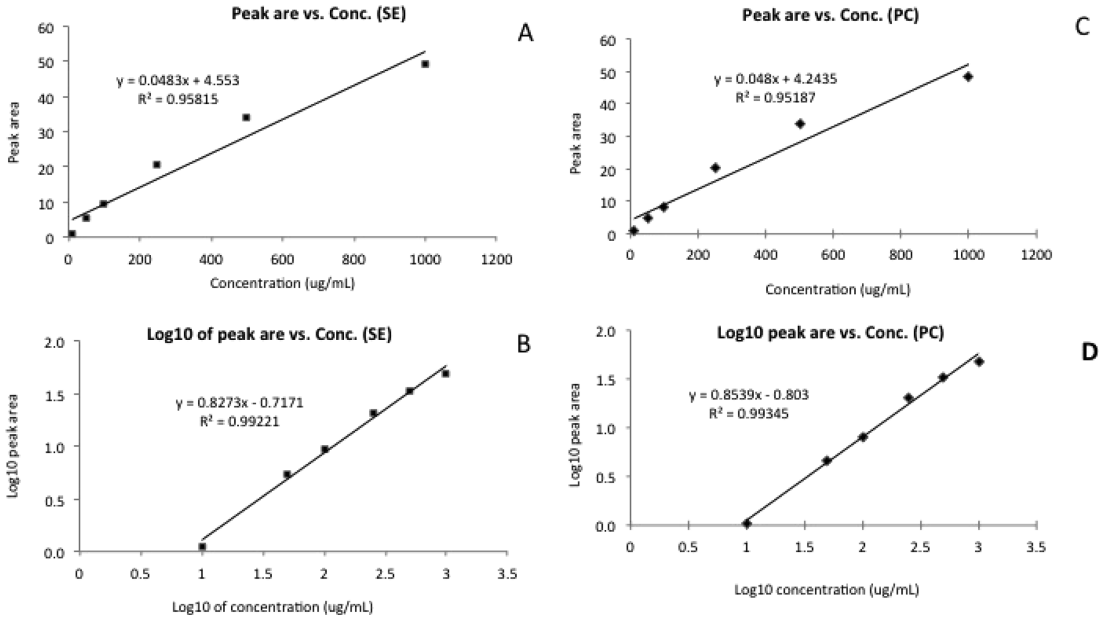

Figure 2. Example calibration curves and response model for lipid analysis by HPLC-CAD Error bars correspond to standard deviation (n = 3).

subsequently the temperature was ramped to 180 °C (25 °C/min) and kept for 1 min, followed by an increase to 220 °C (10 °C/min) and maintained for 1 min. Finally, the temperature was increased to 250 °C (15 °C/min) and held for 3 min. The mass transfer line and ion source were set at 250 and 200 °C, respectively. The FAMEs were detected with electron ionization (70 eV) in a scan mode (50−650 m/z). The identification of unknown FAMEs was achieved by comparing their retention times and mass spectrum profiles with the known standards.

Data Analysis. Results were expressed as mean ± standard deviation, the statistic program for social science (SPSS) software, version 19.0 (SPSS Inc.), was used for statistical analysis. P values < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Development of Chromatographic Separation and Quantification Using HPLC-CAD. Here, we demonstrated the feasibility of analyzing all lipid classes within a single injection using a Luna-HILIC column in combination with a CAD detector. We developed a mixture of three different solvent systems: (A) hexane−acetic acid (99:1, v/v), (B) acetone−isopropanol−acetic acid (29:70:1, v/v), and (C) water−acetone−isopropanol-acetic acid (9:20:70:1, v/v) and performed a gradient-HPLC analysis. With the new solvent system developed in this study, it was possible to analyze at least 11 classes of polar and nonpolar lipids with a single injection. We used CH, which yeast cannot produce, as the spiked internal standard to control the quality of the analysis.

Effect of Column Temperature. We evaluated the effect of column temperature range of 20−50 °C using a mixed standard containing 12 lipid classes. When separation is performed on a HILIC column, the column temperature can affect several separation parameters such as analyte retention, separation efficiency, peak shape, and signal intensity. The chromatographic separation of all lipid classes (Figure 1) can be significantly improved when using a column temperature of 35 °C or higher. The polar and nonpolar lipids were effectively separated and no coelution of polar lipids occurred as observed at low temperatures (at 20 and 30 °C). Column temperature also impacted peak shape as clearly seen in the case of FA analysis, which has a sharper peak shape with increased column temperature. This is because increased temperature increases the diffusion coefficient and results in a narrowing of the analyte peak shape as previously described.[42] Analyte retention was also affected by column temperature as clearly seen in the case of PI for which increasing column temperature resulted in increasing eluting time. Furthermore, a change in analyte retention time is directly related to its signal intensity (case of PI). Since the analyte intensities obtained from the CAD detector depends on the mobile phase composition at the time of analyte elution, the changes in signal intensity were detected when the analyte retention time was shifted. Considering all the effects of column temperature, we selected 35 °C as the optimal for lipid class separation for both polar and nonpolar lipids.

Sample Carryover. Sample carryover is a significant problem when dealing with HPLC separation,[43] the percent column recovery is normally used to evaluate sample carryover. However, it was not possible to determine the column recovery when the analysis was performed on gradient HPLC in combination with the CAD detector as previously discussed. To evaluate sample carryover, we ran a modified gradient-HPLC program that was similar to the program we used for lipid classes separation, but by increasing solvent C to 65% (held for 5 min) instead of the 45% used in a normal run time (data not shown). The increase in solvent C increases the polarity of the system and leads to elution of polar lipids remaining from the previous run. As there were no peaks detected in those test runs, this showed that there was no sample carryover.

Quantitative Analysis Using CAD Detector. Unlike other detectors, such as UV, FID, or ELSD, the relationship between the analyte concentration and CAD response (peak area or peak height) is found to be nonlinear. For example, in the case of SE and PC (Figure 2A and 2C) the value of the correlation coefficient (R^2) was seen to be about 0.95 within the tested concentration range from 10 to 1000 μg/mL. To improve the accuracy of quantification, we therefore used a log−log plot,

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Table 1. Precision (Intra- and Interday), LOD, and LOQ of Individual Lipid Species with HPLC-CAD Method

	peak area (%RSD)		retention time (%RSD)			
compound	intraday (n = 10)	interday[a] (n = 5)	intraday (n = 10)	interday[a] (n = 5)	LOD (μg/mL)	LOQ (μg/mL)
SE	0.63	8	0.002	0.13	1.14	1.53
TAG	1.51	7	0.003	0.12	1.15	1.60
FFA	1.04	12	0.018	1.35	1.15	1.59
CH	1.53	9	0.022	0.57	1.18	1.72
ES	1.25	8	0.011	0.39	1.09	1.34
PA	3.33	10	0.034	0.82	1.91	8.62
CL	3.68	5	0.016	0.31	1.18	1.73
PE	1.01	5	0.003	0.05	1.20	1.81
PC	1.36	4	0.003	0.04	1.15	1.61
SM	4.68	7	0.003	0.04	1.10	1.36
PS	5.88	5	0.005	0.07	1.12	1.47
PI	4.53	7	0.004	0.06	1.14	1.57

[a]Interday precision was determined over the period of 5 days (10 measurements on each day).

which was recommended from the manufacturer to establish a calibration curve and this resulted in a significant improvement of the R^2 values (>0.99) for the SE and PC calibration curves.

Validation and Stability of HPLC-CAD Method. The precision of the HPLC-CAD method was determined by evaluating the repeatability of intraday and interday precision. Intraday precision was determined by repeating the analysis of 500 μg/mL (2 μL injected) standard solution 10 times on the same day. The interday precision was determined over a period of 5 days (10 measurements on each day). The percent relative standard deviation (%RSD) of the peak area (Table 1) were 0.63−5.88% and the retention time were 0.01−1.65% for intraday precision. For interday precision the corresponding % RSD were 4−12% and 0.04−1.35%.

A traditional way to estimate the limit of detection (LOD) and the limit of quantification (LOQ) is to use a signal from blank sample (zero concentration) and standard deviation (SD) from the measurement to provide a conservative value of LOD. This approach is based on the assumption that if the analyte is present, it will produce a signal greater than the noise signal in the absence of the analyte. We have evaluated the LOD using this approach but the estimated LOD values were unreasonable and far from reality. The reason for this is not fully understood, presumably that the detector lacks the ability to distinguish the analytical signal from the noise, especially at very low concentrations. This leads to inconsistency of the measured signal and cause high variation of SD values. To provide a valid analytical signal that can be reliably distinguished from the noise signal a sufficient lipid-standard concentration is required. Therefore, we used the lowest standard concentration of each lipid for the determination of LOD and LOQ. The LOD and LOQ of the instrument were calculated based on $3 \times SD/m$ and $10 \times SD/m$, respectively where SD = standard deviation of 10 time measurements of 10 μg/mL mixed standard (2 μL injected) and m = slope of calibration curve. The LOD (Table 1) for the compounds tested ranged between 1.09−1.91 μg/mL while the LOQ ranged between 1.34−8.62 μg/mL. The estimated LOD and LOQ values using this approach were found to be reasonable and realistic when confirmed by examining the estimated value for the standard containing LOD concentrations. These results indicate that the chromatographic separation developed here is robust, highly reproducible and suitable for the long-term usage.

In conclusion for the HPLC-CAD analysis, the analysis of lipid classes with single injection by HPLC-CAD enables simultaneous analysis of most lipids using one platform. Our results showed that the CAD could be used as a detector for lipid analysis, providing several advantages e.g., capability of direct measurement of most lipid species within a single experiment, high precision and accuracy, and consistent analysis. However, compared with comprehensive lipidomics analysis using chromatography coupled with MS or direct MS shotgun analysis we would like to emphasize that the method developed here is primarily useful for fast and easy screening of many samples. The loss of specificity e.g., molecular information and potentially smaller dynamic range are some of the drawbacks of this detector as compared to MS. Though the method developed in this study covers most lipid species that are found in yeast,[5] it was not able to detect the ceramide lipids in the samples. To address this question we used the ceramide (C18:1n-9) standard to elucidate the retention time to identify the coelution of ceramide with other lipid species. The retention time was found at 21.362 min (data not shown) indicating that ceramide does not coelute with other lipid classes. However, it is not possible with the current system to analyze ceramides in the yeast sample, since the elution time of these lipids are dominated by the noise signal (from 20 to 24 min). Furthermore, ceramide lipids in yeast are normally found only in trace levels. Separate analysis of neutral and polar lipids after the preseparation would be an alternative way for specific analysis of ceramides lipids.

Development of Fast Lipid Extraction Using Microwave Technology. Because of the rigid cell wall of yeast compared to other biological samples, it is more difficult to obtain complete extraction. Therefore, the conventional method[22] for lipid extraction normally involves an additional step as cell disruption (to break or open cell wall) to improve the extraction efficiency. Having two steps of sample preparation is not only time-consuming but increases the possibility of sample loss during the sample preparation process. Additionally, extracting lipids using conventional extraction (liquid−liquid extraction) requires at least 3 h and is normally performed twice to complete the extraction process. Here, we developed an effective extraction method for fast lipid extraction that combines cell disruption and extraction in one step. To provide high throughput for sample preparation, we applied a new approach using microwave technology together with our simple modification of closed-vessel microwave. The extraction was carried out in a commercial Pyrex tube (see more details of experimental set up from our previous work[29]).

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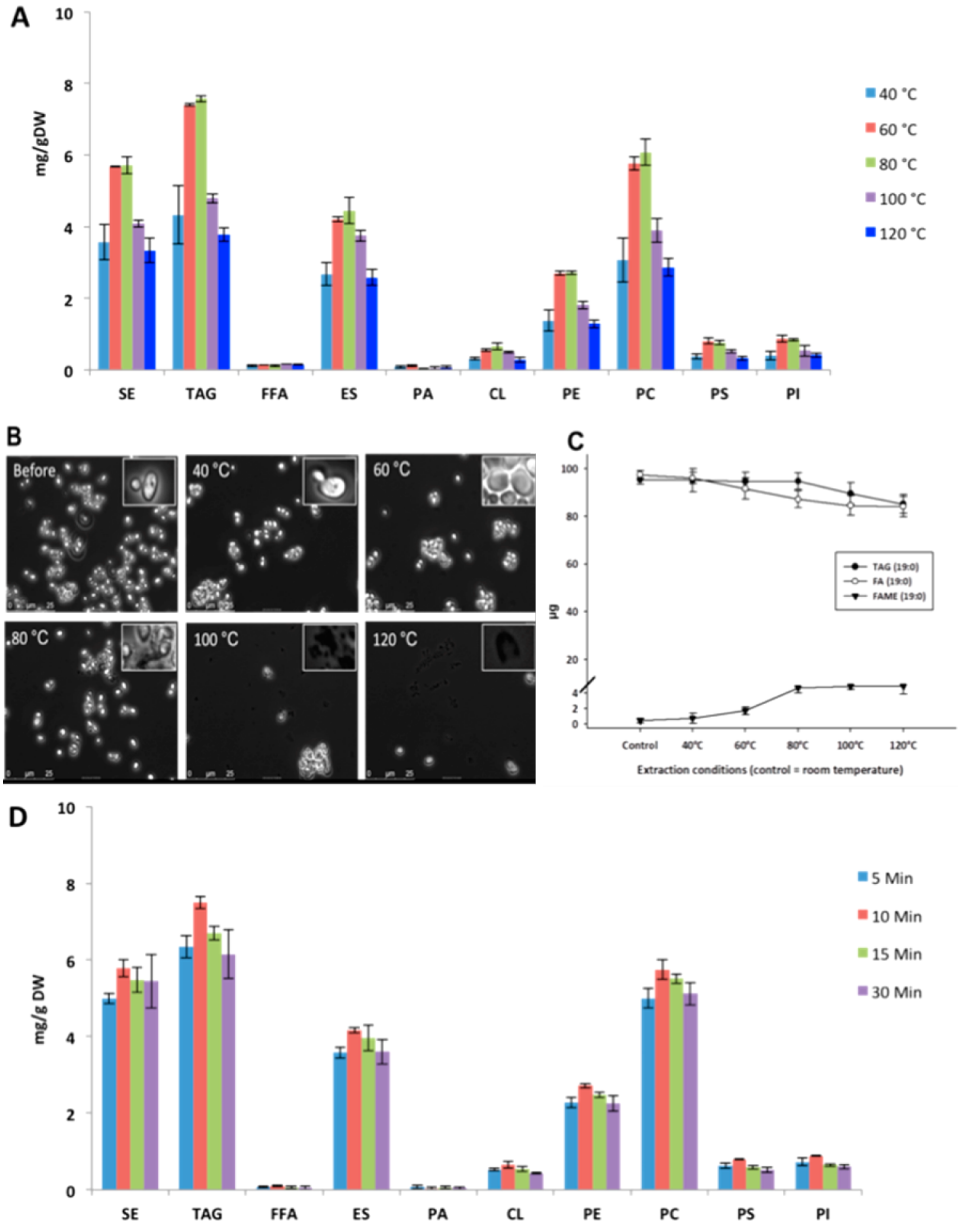

Figure 3. Optimizing extraction parameters. (A) Extraction yields of lipid classes, all reactions were performed in 10 min. Cholesterol was used as internal standard. (B) Microscopic results showing the effect of different extraction temperatures on cell disintegration. (C) Heat-induced esterification. The bound fatty acid standard (TAG, 19:0) and free fatty acid (FA) were spiked into the blank extraction solvent (TAG and FA were detected by HPLC-CAD and FAME was quantified by GC-MS). All reactions were performed in 10 min. (D) Optimizing extraction duration. All reactions were performed at 60 °C. Cholesterol was used as internal standard. Error bars correspond to standard deviation (n = 3).

The benefit of this is an increased rate of sample preparation (up to several hundred per day), which can facilitate large-scale studies.

Microwave-assisted extraction uses microwave energy to heat the solvent in contact with the sample and analytes.[26,44] Parameters, such as solvent types, solvent volume, extraction time, or temperature, directly influence the extraction efficiency. The solvent choices and solvent volumes used in this study were fixed according to conventional protocols.[22] We investigated the effect of extraction temperature and extraction time, because these two factors directly influence the extraction yields of lipids.

Optimization of the Extraction Temperature. The extraction temperature was evaluated in the range of 40−120 °C with 10 min as fixed extraction time. The temperatures of 60 and 80 °C (Figure 3A) were found to be the optimal points as evidenced by the highest yields for most lipids compared to other temperatures. An increase in temperature to more than 80 °C resulted in decreased yields of some lipids, and this could possibly be due to degradation or changes in the original structure of some lipid species as previously reported in the case of SE.[45]

As the cell disruption step can improve the extraction efficiency, most of the conventional methods used for lipid extraction in yeast samples. We observed from the microscopic results (Figure 3B) that the structures of yeast cells were not changed when cells were extracted at low temperature (40 °C, Figure 3B) as compared with the control (Figure 3B; yeast cells with no extraction), as cellular compartments were still visible after the extraction process. At 60 °C, yeast cells seem to be

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disintegrated and the subcellular structures were not found inside the cells. This indicated the leakage and infusion of extraction solvent into the cells and this could result in improved extraction efficiency. However, we started to see some cell debris caused by overheating at high temperature (80 °C), and much damaged cell debris were found when higher temperatures (100−120 °C) were used for microwave-assisted extraction.

In the presence of methanol in the extraction solvent, the esterification of bound or free fatty acids can occur during the extraction process and this will result in yield loss of extracted lipids (by heat-induced esterification). To address this question, we extracted the standard mixture of TAG (19:0) and FA (19:0) with microwave extraction at temperatures from 40 to 120 °C with 10 min as fixed reaction time. We also performed negative control by extracting the same standard mixture using conventional protocols, which were extracted at room temperature (details in Conventional Lipid Extraction section). After the extraction process, the extracted standards were equally divided in to two parts and measured separately. One part was measured with HPLC-CAD to detect TAG (19:0) and FA (19:0) and another part was used to directly measure FAME (19:0) by GC-MS. It was observed (Figure 3C) that the yields of TAG (19:0) decreased by about 10−15% when using extraction temperatures of 100 or 120 °C. Similarity to the case of FA (19:0), the yield of FA was decreased 10−15% when using an extraction temperature between 80 and 120 °C. In general, free fatty acids require milder conditions for esterification compared to bound fatty acids. Therefore, the effect of temperature on FAME reaction was found with spiked FA (19:0) more than with spiked TAG (19:0). The results obtained from HPLC-CAD were consistent with results obtained from GC-MS when measuring an increase of FAME at different extraction temperatures. On the basis of the extraction efficiency and heat-induced esterification, we selected the extraction temperature at 60 °C to be the optimal condition for microwave extraction and used this for further study.

Optimization of the Extraction Time. The optimal extraction time of lipids depends on type and size of the sample. A sample in complex matrices may require longer extraction time. To identify the optimal reaction time, we performed lipid extraction by microwave extraction in a range from 5 to 30 min at 60 °C, using a sample of approximately 10 mg. Results (Figure 3D) showed that the optimal extraction times of most lipid classes were found by using 10 min whereas performing longer extraction times did not significantly increase extraction yields of the lipids. We therefore selected 10 min as the optimal extraction time.

Validation of Microwave-Assisted Extraction Method. We validated the new extraction method by comparing it with conventional extraction. For the conventional method, the cell disruption step (by glass beads) was added to the process prior the total lipid extraction to obtain the highest efficiency of extraction method. The CH standard was spiked into samples used for both extraction methods. On the basis of the recovery of the spiked CH standard, the efficiencies of total lipid extraction obtained from the two methods were found to be equally effective. There was an insignificant difference ($P > 0.05$) in percent recovery of CH for the two extraction methods, that is, 92 ± 6% for the conventional and 93 ± 8% for the microwave method ($n = 3$). The high recovery of CH internal standard in both methods indicated that both methods are highly effective for extraction of lipids in yeast cells. There were also no significant differences ($P > 0.05$) in yields of the different lipid species obtained with both conventional extraction and microwave-assisted extraction (Figure 4). This

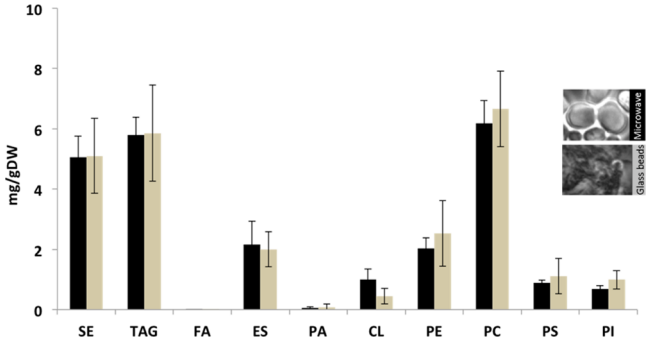

Figure 4. Comparison of microwave (10 min at 60 °C) and conventional method (3 h. at room temperature). Error bars correspond to standard deviation ($n = 14$).

indicated that the method developed from this study was as efficient as the conventional extraction method. On the other hand, the reproducibility (observed from the standard deviations as error bars in Figure 4) for all extracted lipids was significantly lower with the microwave-assisted extraction compared with the conventional method. Presumably, this is the result of the nonhomogenous cellular disruption obtained when using glass beads with the conventional method. We demonstrated here that the extraction of lipids in yeast cells using microwave technology provided the same extraction efficiency as compared to the conventional method. Reducing the extraction time from 360 min (conventional) to 10 min, and combining cell disruption and extraction in one step are therefore a clear advantage of the new method over the conventional method.

CONCLUSIONS

Since *S. cerevisiae* has been established to use as a cell factory for the production of biofuels and several biochemical products, focus on engineering its lipid metabolism has recently increased, and a high-throughput method for fast screening of different lipids during the development process is therefore highly desirable. The methods developed and presented here will likely become useful tools to support this need.

ASSOCIATED CONTENT

Supporting Information

Identification of unknown lipids, additional methods, confirmation of ergosterol, confirmation of free fatty acids, and additional references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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