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Development and validation of a hydrophilic interaction liquid chromatography–tandem mass spectrometry method for the quantification of lipid-related extracellular metabolites in *Saccharomyces cerevisiae*

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ABSTRACT

A highly sensitive hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) method was developed and validated for the quantification of glycerophosphoinositol (GroPIns), glycerophosphocholine (GroPCho), glycerol 3-phosphate (GroP), inositol, and choline in the extracellular medium of *Saccharomyces cerevisiae*. The media samples were pretreated with a single two-phase liquid extraction. Chromatographic separation was achieved on a Waters Xbridge HILIC (150 mm × 4.6 mm, 5 μm) column under isocratic conditions using a mobile phase composed of acetonitrile/water, 70:30 (v/v) with 10 mM ammonium acetate (pH adjusted to 4.5) at a flow-rate of 0.5 mL/min. Using a triple quadrupole tandem mass spectrometer, samples were detected in multiple reaction monitoring (MRM) mode via an electrospray ionization (ESI) source. The calibration curves were linear ($r^2 \geq 0.995$) over the range of 0.5–150 nM, with the lower limit of quantitation validated at 0.5 nM for all analytes. The intra- and inter-day precision (calculated by coefficient of variation, CV%) ranged from 1.24 to 5.88% and 2.46 to 9.77%, respectively, and intra- and inter-day accuracy (calculated by relative error, RE%) was between −8.42 to 8.22% and −9.35 to 6.62%, respectively, at all quality control levels. The extracellular metabolites were stable throughout various storage stability studies. The fully validated method was successfully applied to determine the extracellular levels of phospholipid-related metabolites in *S. cerevisiae*.

1. Introduction

The metabolome represents the full collection of low-molecular weight chemical species within a cell or biological system, and is considered the endpoint of "omics analysis" [1–3]. Current research on metabolomic investigation consists of four complementary approaches: target analysis, metabolic profiling, metabolic fingerprinting, and metabolic footprinting [4–6]. All of these approaches are usually applied to investigate differences in metabolite concentrations after alterations in the biological environment or upon genetic modification. The measurement of extracellular metabolites secreted from the intracellular volume into the growth medium is termed "metabolic footprinting". Footprinting analysis offers important technical advantages over the analysis of intracellular compounds, referred to as "metabolic fingerprinting" [7–9]. First of all, the extracellualar metabolome is generally quite stable owing to the relative lack of enzymes that can convert the metabolites into other products. Therefore, the time-consuming quenching steps associated with the analysis of intracellular metabolites are not required when extracellular metabolites are analyzed. In addition, the extracellular metabolome is generally simplified in terms of the number of metabolites present and their concentration ranges, as compared to the intracellular metabolome [7]. Metabolic footprinting has been used in the classification of microbial mutants for functional genomics studies by employing mass spectrometry (MS) [3,10–12] or nuclear magnetic resonance (NMR) analysis [13]. For yeast and mammalian cells, metabolic footprinting has been performed under a limited number of conditions, and has primarily focused on central carbon metabolites [14–18]. To our knowledge, no metabolic footprinting study has focused on lipid metabolites.

The simple eukaryote, *Saccharomyces cerevisiae*, has been used as a model to study many aspects of cell biology, including lipid

Abbreviations: CV, coefficient of variation; GroPCho, glycerophosphocholine; GroPIns, glycerophosphoinositol; GPXs, glycerophosphodiesters; GroP, glycerol 3-phosphate; HILIC, hydrophilic interaction liquid chromatography; HQC, high quality control; LLOQ, lower limit of quantification; LQC, low quality control; MRM, multiple reaction monitoring; MQC, middle quality control; PLB, phospholipases of the B type; QC, quality control; RE, relative error.

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metabolism [19–23]. We have chosen to analyze five major phospholipid metabolites in the media of *S. cerevisiae* as a means of monitoring aspects of phospholipid synthesis and turnover in the organism: inositol, choline, glycerol 3-phosphate, glycerophosphoinositol, and glycerophosphocholine. The lipid precursors inositol and choline regulate phospholipid biosynthesis at the transcriptional level in *S. cerevisiae* [24]. Thus, they are frequently added to and removed from medium to study and manipulate the biosynthetic pathways. Inositol and choline can also be produced by the cell via lipid turnover events. GroP is a metabolite that can be produced through phospholipid turnover and is also a precursor involved in phospholipid biosynthesis. Finally, extracellular GroPIns and GroPCho are produced through the hydrolytic cleavage of both acylester bonds of plasma membrane-associated glycerophospholipids by phospholipase B type enzymes [20,21,25]. Thus, extracellular GroPIns and GroPCho can be monitored as an indicator of phospholipase B activity encoded by the *PLB1*, *PLB2*, and *PLB3* genes. Overall, the concentration of each of these metabolites in the medium is the result of two processes: their production via enzymatic activity and their uptake via plasma membrane transport. By knowing the extracellular levels of these metabolites, we will gain insight into the status of the phospholipid biosynthetic and catabolic pathways in *S. cerevisiae*.

This method was developed in order to create a simplified and sensitive method for the absolute and simultaneous quantitation of the levels of important phospholipid metabolites in the media of *S. cerevisiae*. Previous studies in which one or more of the compounds has been monitored usually involve the use of radioactivity, as the compounds have no UV or fluorescence detectable groups. For example, ^{3}H or ^{14}C labeled inositol or choline, and ^{32}P-H_3PO_4 are typically utilized [26–28]. However, measuring all of these compounds simultaneously with multiple radioactive compounds is technically challenging, even if a good separation system is at hand. In addition, the use of radioactive molecules can also present a quantitation problem. For example, since *S. cerevisiae* can both synthesize and import inositol and choline, but radioactive labeling only allows for the discrimination of inositol and choline transported into the cell, not that derived from de novo synthesis. In contrast, MS detects all chemical species, regardless of their mode of synthesis. Finally, the method described here is an advance in terms of the LC separation of the compounds. While published procedure has described the chromatographic separation of choline-containing metabolites, and inositol-containing metabolites, we know of no published procedure for the LC separation of all five of these compounds simultaneously [29–31].

Two published studies are pertinent to the method described here. A LC–MS/MS method using a β-cyclodextrin-bonded column was described for the quantitative analysis of only internal GroPIns in rat cell lines, but other metabolites were not included in this method [32]. In contrast, our method analyzes five lipid-related metabolites, including GroPIns, in the extracellular medium of *S. cerevisiae*. Another study used normal phase LC–MS to quantitate several water-soluble metabolites, including GroPIns and GroPCho, from rat brain tissue [33]. Our method differs from that of Kopp et al. [33] and Dragani et al. [32] in that we perform extensive method validation, we analyze an overlapping but different set of metabolites, and we use HILIC chromatography, which results in decreased retention times as compared to normal phase.

Here, we present a hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) method for the quantification of five lipid-related extracellular metabolites in *S. cerevisiae* cells. A liquid–liquid extraction procedure has been combined with an extensive optimization of HILIC–MS/MS methodology to provide effective and reliable chromatographic separation of the analytes. The method is highly sensitive and has been thoroughly validated according to the bioanalytical method validation guidelines for industry as specified by the US Food and Drug Administration (FDA) (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf).

2. Experimental

2.1. Materials and reagents

Standard compounds including glycerophosphoinositol (L-α-glycerophospho-D-myo-inositol), glycerophosphocholine (L-α-glycerophosphorylcholine), glycerol 3-phosphate (L-α-glycerophosphate), myo-inositol, and choline chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). The deuterated internal standard (IS), choline chloride-d_9, was obtained from Cambridge Isotopes (Andover, MA, USA). Ammonium acetate was purchased from Fisher Scientific (Pittsburgh, PA, USA). Optima grade methanol and acetonitrile used for LC–MS/MS analysis were also purchased from Fisher Scientific. Deionized water was obtained using a Milli-Q water purification system from Millipore (Bedford, MA, USA).

2.2. Preparation of standard solutions and quality control samples

Primary stock solutions of glycerophosphoinositol (GroPIns), glycerophospho-choline (GroPCho), glycerol 3-phosphate (GroP), myo-inositol, choline chloride, and choline chloride-d_9 (IS) were prepared in methanol/water, 1:1 (v/v) at a concentration of 50 mM, respectively. Working standard solutions were made by diluting the primary stock solutions in water to a concentration of 10 μM. A working standard solution of the internal standard (choline-d_9) in 2.5 μM was prepared by diluting its primary stock solution in deionized water. Eight calibration standards were prepared by adding 25 μL aliquots of appropriate dilution of the working standard solutions and internal standard to 475 μL aliquots of the blank medium to obtain the following concentrations: 0.5, 1.5, 5, 10, 30, 60, 90 and 150 nM (internal standard was at 125 nM). Quality control (QC) samples were prepared at concentrations of 2 nM (low QC, LQC), 50 nM (middle QC, MQC), and 100 nM (high QC, HQC) by spiking the blank media with the working standard solutions. The primary stock solutions, working standard solutions and QC samples were stored at −20 °C until use.

2.3. Culture conditions and sample preparation

The *S. cerevisiae* wild type strains, BY4741 (JPV203, *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (JPV399, *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) were purchased from Open Biosystems (Thermo Scientific, Huntsville, AL, USA). The deletion strains were constructed using standard homologous recombination techniques. Drug resistant markers, *hph*, *KanMX*, and *nat1* were amplified from plasmids pAG32, pUG6, and pAG25, respectively, and were inserted in place of the target genes for deletion. The plasmids were received from Euroscarf [34,35]. Deletion strains were constructed in JPV203 to produce *plb1::hph* (JPV617), *plb2::KanMX* (JPV618), *plb3::nat1* (JPV 619), *plb1::hph/plb2::KanMX/plb3::nat1* (JPV623), and in strain JPV399 to produce *plb1::hph* (JPV668). Strains were grown aerobically at 30 °C in yeast nitrogen base (YNB) medium containing 75 μM inositol, 20 μM choline, and 2 mM KH_2PO_4 instead of normal 7.35 mM KH_2PO_4 in order to minimize ion suppression in mass spectrometry. Growth was monitored by measuring the OD 600 nm using a Biomate 3 Thermo Spectronic spectrophotometer.

To prepare medium samples, culture aliquots were centrifuged at 3000 rpm for 5 min, and the resulting supernatant was filtered. A

liquid–liquid extraction approach was performed in order to separate polar lipid metabolites from the non-polar materials in the extracelluar medium. 10 μL of internal standard working solution and 100 μL aliquots of the metabolite containing supernatant were added to 890 μL of chloroform/methanol, 2:1 (v/v). This mixture was vortexed for 2 min, followed by centrifugation at 3000 rpm for 3 min. A 200 μL aliquot of the upper phase (49.3 ± 1.0% of the total analytes found in the upper phase, since the total upper phase volume was approximately 400 μL) was collected and transferred into a 1.5-mL HPLC vial for evaporation under nitrogen. The residue was reconstituted in 200 μL of acetonitrile/methanol, 75:25 (v/v) and was diluted in 1:100 fold volumes before analysis, in order to remain within the calibration range. Less dilution could be used to increase the sensitivity of the method. The sample injection volume to LC–MS/MS system was 10 μL.

2.4. Instrumentation

Chromatographic studies were performed via a 1200 series Rapid Resolution LC system coupled with a 6440 Triple Quadrupole Mass Spectrometer (Agilent, Santa Clara, CA, USA). MassHunter workstation software package was used for data acquisition and processing. Quantification was determined using qualitative (B1.0.82.2) and quantitative (B1.0.77.3) analysis as implemented in the MassHunter software.

2.4.1. Chromatographic conditions

The analytical column used was a Waters Xbridge HILIC (150 mm × 4.6 mm, 5 μm) column from Waters Corporation (Milford, MA, USA). Separations were performed under isocratic conditions at a flow rate of 0.5 mL/min at room temperature. Mobile phase consisted of acetonitrile/water, 70:30 (v/v) with 10 mM ammonium acetate adjusted to pH 4.5 with glacial acetic acid. Column effluent was then passed through the mass spectrometer for later detection.

2.4.2. MS settings

Electrospray mass spectrometry (ESI-MS) was performed on a triple quadrupole mass spectrometer. Mass transitions of m/z for each metabolite and IS were optimized by direct infusion of the respective analytes in acetonitrile/water, 70:30 (v/v) with 10 mM ammonium acetate in product ion mode. Optimal settings for subsequent LC–MS analyses were manually adjusted. Metabolites were detected in both positive and negative ionization modes at different time segments in one run using multiple reaction monitoring (MRM). Other MS parameters were set as follows: capillary voltage of 3.5 kV, ion source temperature of 300 °C, cone voltage of 50 V, and nebulizer gas temperature of 250 °C. Cone and evaporation gas flow rates (both are nitrogen) were set at 600 and 480 L/h, respectively. Low and high mass resolutions were fixed at 15 (arbitrary units) for both the first and third quadrupole mass analyzers. Dwell time and interchannel delay were maintained at 200 and 10 ms, respectively. Collision energy in the rf-only quadrupole cell and transitions were optimized for each metabolite on the reference compound (Table 1).

2.5. Method validation

The LC–MS/MS method was validated through evaluation of specificity, linearity, sensitivity, intra- and inter-day precision, accuracy, recovery, and stability in accordance with currently approved FDA bioanalytical method validation guidelines.

2.5.1. Specificity and selectivity

To determine if endogenous contaminants were present in coelution with the analytes, the chromatography was developed and the MS/MS parameters were optimized to obtain separation of the analytes from potentially interfering compounds as well as the highest signal to noise ratios.

2.5.2. Linearity of calibration standards, precision, and accuracy

Linearity was evaluated over the concentration range of 0.5–150 nM. The calibration curve was constructed by plotting the peak area ratio of analyte to that of IS against the nominal concentration of calibration standards. The results were fitted to least-squares linear regression analysis with a correlation coefficient of 0.995 or better. Intra-day and inter-day precision and accuracy were determined through analysis of six replicates at four different levels (0.5, 2, 50 and 100 nM) during the same day and on three different days. The precision was determined by calculating the coefficient of variation (CV), and the accuracy was expressed in the form of the relative error (RE), which was calculated as the percent deviation of the mean from the true value. The acceptance criteria of the data included precision within ±15% CV from the nominal values and an accuracy of within ±15% RE, except for LLOQ, where it should not exceed ±20% of accuracy as well as precision (bioanalytical method validation guidelines of FDA).

2.5.3. Recovery

The extraction recoveries of analytes from medium were determined by comparing the mean peak areas of medium spiked with standards at three levels (2, 50 and 100 nM, $n = 6$) to those of blank medium extracts spiked with standards at equivalent concentrations, corrected for the evaporated volume of organic phase.

2.5.4. Matrix effect

Matrix effects from the medium samples were evaluated by comparing the peak areas of the spiked blank medium extracts (2, 50 and 100 nM, $n = 6$) to the peak areas from standards spiked in mobile phase of acetonitrile/water, 70:30 (v/v) with 10 mM ammonium acetate.

2.5.5. Stability

The stability of analytes in medium was investigated using QC samples (2 and 100 nM) stored under different temperature conditions for different periods of time. The six replicates were subjected to three freeze (12, 18 and 24 h)–thaw (2, 3 and 4 h) cycles. Short-term storage stability was evaluated at room temperature for 4 h, and long-term storage stability at −20 °C for 30 days. Reinjections of QC samples ($n = 6$) were analyzed after 24 h storage at the same concentrations in the autosampler.

3. Results and discussion

3.1. Optimization of MS/MS conditions

The standards of the analytes (Fig. 1) were first characterized by total ion scan and product ion scan through direct infusion to ascertain their precursor ions and to select product ions for use in MRM mode. The MS/MS parameters, specifically fragmentor voltage and collision energy, were evaluated for the best response of the parent ion and daughter ion, respectively, using the automatic optimization process and then transferred to the MRM method. GroPCho, GroP, and choline were found to give the most intense protonated molecular ions under positive ionization, while GroPIns and inositol were detected in negative ionization mode. Full scan spectra produced a predominant peak for $[M+H]^+$ at m/z 258.0 of GroPCho, 172.7 of GroP, 104.1 of choline, and 113.1 of internal standard, respectively. The deprotonated molecular ions $[M-H]^-$ of GroPIns and inositol were detected at m/z 333.0 and 178.7, respectively. Collision induced dissociation of the precursor ions produced

Table 1
Tandem mass spectrometry parameters for transition pairs in the MRM mode.

Analyte	Ionization mode	Precursor ion	Transition pairs	Collision energy (eV)
Choline	Positive	104.1	104.1 → 60.1	10
Choline-d_9	Positive	113.1	113.1 → 69.1	10
GroP	Positive	172.7	172.7 → 98.8	10
Inositol	Negative	178.7	178.7 → 86.8	20
GroPCho	Positive	258.0	258.0 → 104.0	10
GroPIns	Negative	333.0	333.0 → 152.9	20

major fragmentation for each analyte. Therefore, the transition pairs 258.0 → 104.0, 172.7 → 98.8, 104.1 → 60.1, and 113.1 → 69.1 were optimized for GroPCho, GroP, choline, and choline-d_9, respectively (Fig. 2). Using similar procedures, the MRM transition of GroPIns and inositol were determined to be 333.0 → 152.9, and 178.7 → 86.8, respectively (Fig. 2).

3.2. Optimization of LC conditions

Polar lipid-related metabolites were extracted from the extracellular medium by a liquid–liquid extraction. Chloroform/methanol, 2:1 (v/v) was used as the extraction solvent to remove non-polar compounds from the medium. The choice of a chromatography method was less straightforward since a diverse range of metabolites at widely differing concentrations are present in culture medium. A HILIC column was chosen as an appropriate approach based on a review of the literatures for smaller hydrophilic and ionizable analytes [36–45]. A Waters Xbridge HILIC (150 mm × 4.6 mm, 5 μm) column was selected to provide the best compromise between specificity and speed of analysis. With the optimal isocratic elution, the metabolites were adequately separated with retention times of 5.58 min for inositol, 15.47 min for GroP, 15.68 min for GroPIns, 5.39 min for GroPCho, and coelution of choline and choline-d_9 at 3.38 and 3.37 min, respectively (Fig. 3B). The retention times varied by less than 0.01 min from run to run under these conditions.

3.3. Method validation

3.3.1. Specificity and selectivity

Representative chromatograms of blank medium, a spiked medium sample, and the medium obtained after growth of a wild

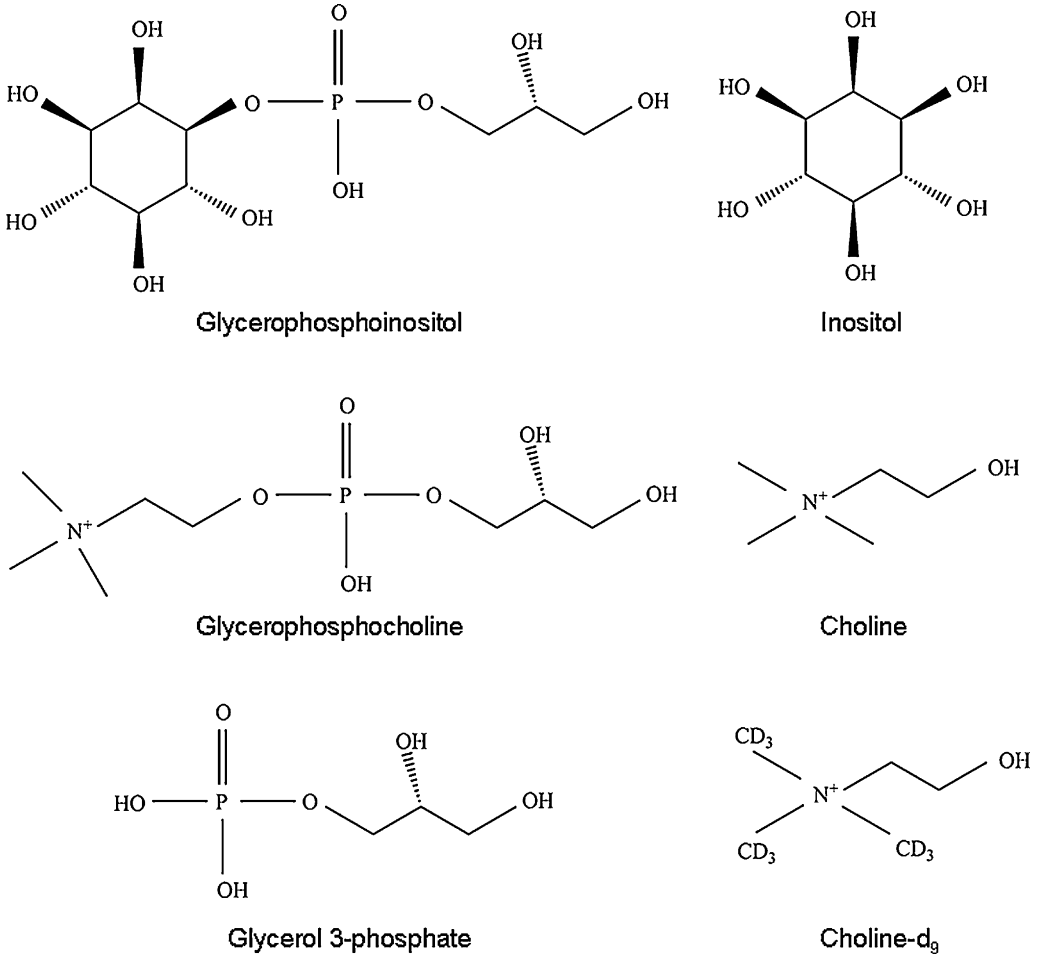

Fig. 1. Molecular structures of GroPIns, GroPCho, GroP, inositol, choline, and choline-d_9.

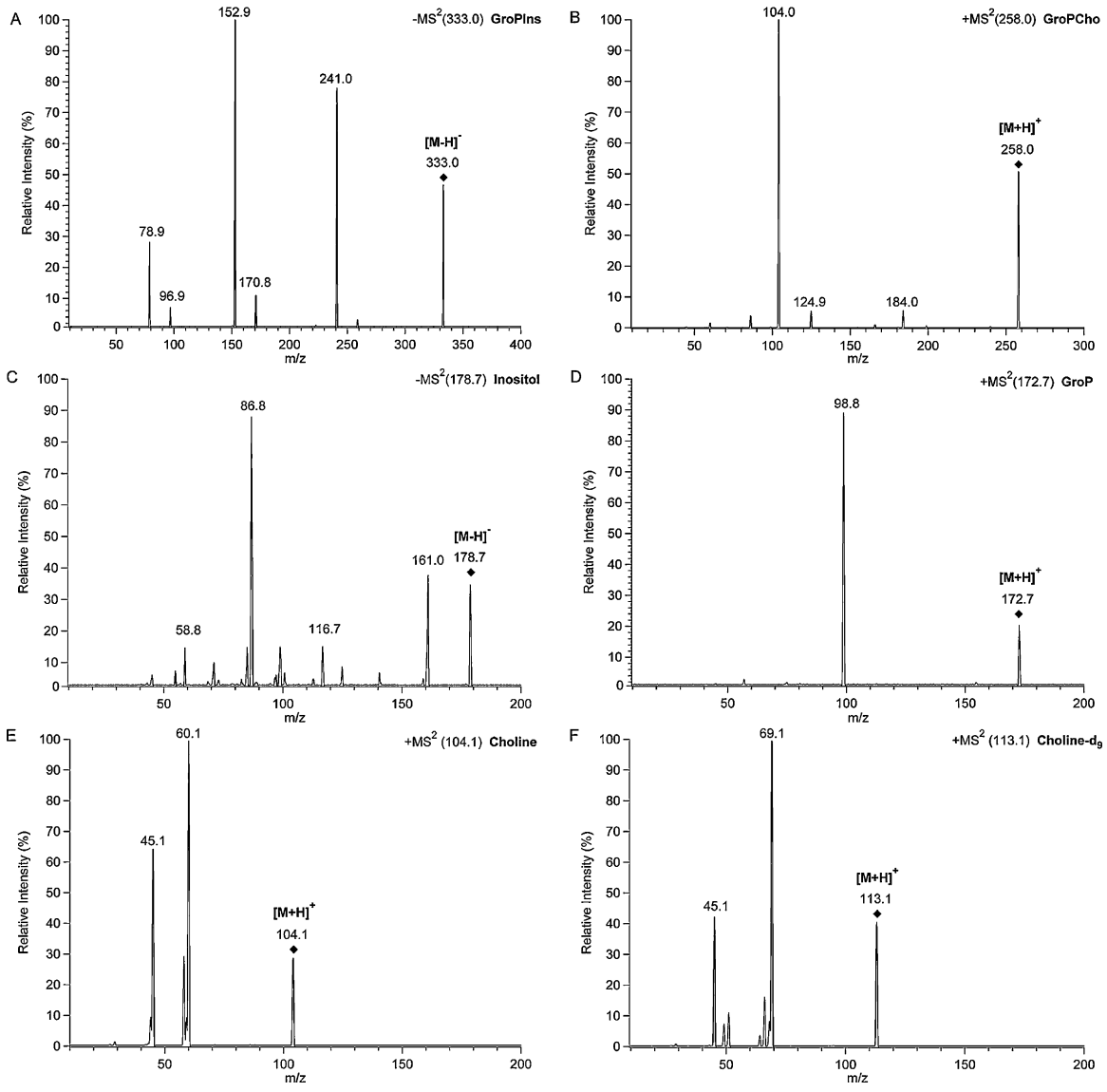

Fig. 2. Tandem mass spectra of (A) GroPIns, (B) GroPCho, (C) inositol, (D) GroP, (E) choline, and (F) choline-d_9.

type strain were shown in Fig. 3. No interference with the retention time of the analytes and internal standard (Fig. 3B and C) was observed in the blank medium (Fig. 3A), indicating the specificity of the present method.

3.3.2. Linearity of calibration and LLOQ

The calibration curves were constructed by plotting the instrument response (peak area ratio (analyte/IS)) versus calibration standard concentration. A linear relationship was constructed over the concentration range of 0.5–150 nM. Correlation coefficients (r^2) of the calibration curves for all standards ranged between 0.995 and 0.997. The precisions (CV%) were less than 8.7%, and the accuracies (RE%) were within the range of −6.2 to 8.3% in Table 2. Using the present method, the LLOQ was validated at 0.5 nM for all analytes, which is equivalent to 186, 129, 185, 90 and 70 pg/mL for GroPIns, GroPCho, GroP, inositol, and choline, respectively. Under the present LLOQ, the concentration of analyte in the medium can be determined even 24 h after administration. As a result, this method was sensitive enough to investigate the levels of these metabolites in the exometabolome of *S. cerevisiae*.

3.3.3. Precision and accuracy

Table 3 is a summary of the intra- and inter-day precision and accuracy validation using QC samples as described in Section 2.5. QC samples were determined in replicate ($n = 6$) at three different concentrations: 2, 50, and 100 nM. Within the range of 2–100 nM, the intra-day and inter-day precisions (CV%) were between 1.2 and 9.8% for each QC level of analytes. The accuracy (RE%), determined for QC samples, was within −9.3 to 8.3% for each QC level of analytes.

3.3.4. Recovery

The mean extraction recoveries of analytes in Table 4 were 74.4% for GroPIns, 72.6% for GroPCho, 80.7% for GroP, 82.5% for inositol, 75.9% for choline, and 75.5% for IS at 50 nM. The simple sample extraction procedure with 10 volumes of chloroform/methanol, 2:1 (v/v) to 1 volume of sample allowed for high sample throughputs and good recoveries. No significant degradation occurred during the extraction procedure.

3.3.5. Matrix effect

The absolute matrix effects, the ratio of the peak areas of three-concentration level standards spiked in medium extracts to areas of

Table 2
Precision and accuracy of calibration samples[a].

Analyte	Nominal concentration (nM)							
	0.5	1.5	5	10	30	60	90	150
GroPIns								
Mean	0.47	1.52	5.06	9.73	31.84	63.92	94.21	146.62
Precision (CV%)[b]	3.44	2.79	6.82	5.40	4.27	6.98	3.18	4.02
Accuracy (RE%)[c]	−6.24	1.33	1.24	−2.75	6.13	6.53	4.68	−2.25
GroPCho								
Mean	0.50	1.47	4.93	10.44	30.37	62.82	92.29	145.22
Precision (CV%)	4.29	8.70	3.37	6.13	4.28	3.27	4.91	2.47
Accuracy (RE%)	0.03	−2.73	−1.42	4.42	1.23	4.75	2.54	−3.19
GroP								
Mean	0.52	1.46	5.09	9.52	29.64	57.28	88.74	146.25
Precision (CV%)	4.25	1.57	3.81	2.39	5.07	2.73	4.20	3.31
Accuracy (RE%)	4.20	2.67	1.83	−4.88	1.21	−4.53	−1.45	−2.58
Inositol								
Mean	0.46	1.58	5.12	10.67	30.88	58.73	93.64	147.27
Precision (CV%)	2.55	4.98	6.96	3.38	5.26	3.01	2.29	4.47
Accuracy (RE%)	8.31	5.33	2.46	6.70	2.93	−2.12	4.04	−1.82
Choline								
Mean	0.51	1.54	4.85	9.82	29.64	62.49	85.26	145.72
Precision (CV%)	3.01	6.28	4.11	5.26	4.42	1.57	2.06	6.31
Accuracy (RE%)	2.11	2.67	−3.31	−1.87	−1.22	4.15	−5.27	−2.85

[a] $n = 6$ for each of 3 runs.
[b] [Standard deviation/mean concentration measured] × 100.
[c] [(Mean concentration measured − nominal concentration)/nominal concentration] × 100.

Table 3
Intra- and inter-day precision and accuracy of the QC samples.

Analyte (nM)	Intra-day ($n = 6$)				Inter-day ($n = 18$)			
	Mean	SD	PRE[a] (CV%)	ACC[b] (RE%)	Mean[c]	SD	PRE (CV%)	ACC (RE%)
GroPIns								
2	2.04	0.12	5.88	2.16	1.92	0.09	4.69	−4.64
50	48.37	1.46	3.02.	−3.26	53.25	3.64	6.84	6.52
100	92.66	2.27	2.45	−7.34	90.66	5.82	6.42	−9.35
GroPCho								
2	1.97	0.09	4.57	−1.58	2.05	0.11	5.37	2.51
50	52.01	2.42	4.65	4.02	47.62	1.17	2.46	−4.76
100	94.67	1.17	1.24	−5.33	92.13	3.87	4.20	−7.87
GroP								
2	1.89	0.07	3.70	−5.53	1.94	0.14	7.22	−3.68
50	51.39	1.98	3.85	2.78	53.22	4.21	7.91	6.44
100	96.64	4.61	4.77	−3.36	95.27	2.36	2.48	−4.73
Inositol								
2	2.07	0.11	5.31	3.54	1.97	0.14	7.11	−3.74
50	47.65	2.61	5.48	−4.77	47.29	4.62	9.77	−5.42
100	91.58	3.52	3.84	−8.42	94.63	3.10	3.28	−5.37
Choline								
2	1.92	0.06	3.13	−4.65	1.95	0.09	4.62	−2.52
50	54.11	1.29	2.38	−8.22	46.74	2.73	5.84	−6.53
100	94.28	4.17	4.42	−5.72	92.18	4.71	5.11	−7.82

Abbreviations: PRE, precision; ACC, accuracy.
[a] [Standard deviation/mean concentration measured] × 100.
[b] [(Mean concentration measured − nominal concentration)/nominal concentration] × 100.
[c] $n = 3$ days with six replicates per day.

Table 4
Absolute matrix effect and recovery of all analytes at three concentrations of 2, 50 and 100 nM.

Analyte	Absolute matrix effect[a] (%)				Recovery[b] (%)			
	LQC	MQC	HQC	Mean	LQC	MQC	HQC	Mean
GroPIns	97.2	94.3	95.3	95.6	72.5	75.1	75.6	74.4
GroPCho	97.4	95.9	99.8	97.7	70.5	72.9	74.4	72.6
GroP	98.5	97.6	99.0	98.4	81.7	78.4	82.0	80.7
Inositol	94.2	96.6	95.1	95.3	82.9	83.3	81.3	82.5
Choline	98.6	95.1	97.3	97.0	73.6	77.9	76.2	75.9

[a] Absolute matrix effect expressed as the ratio of the mean peak areas of three-concentration level standards spiked in medium extracts to those from standards spiked in the mobile phase multiplied by 100.
[b] Recovery calculated as the ratio of mean peak areas of extracts prepared from blank medium spiked with standards to those of blank medium spiked with standards without an extraction procedure multiplied by 100. The standards were used at three levels (2, 50 and 100 nM, $n = 6$).

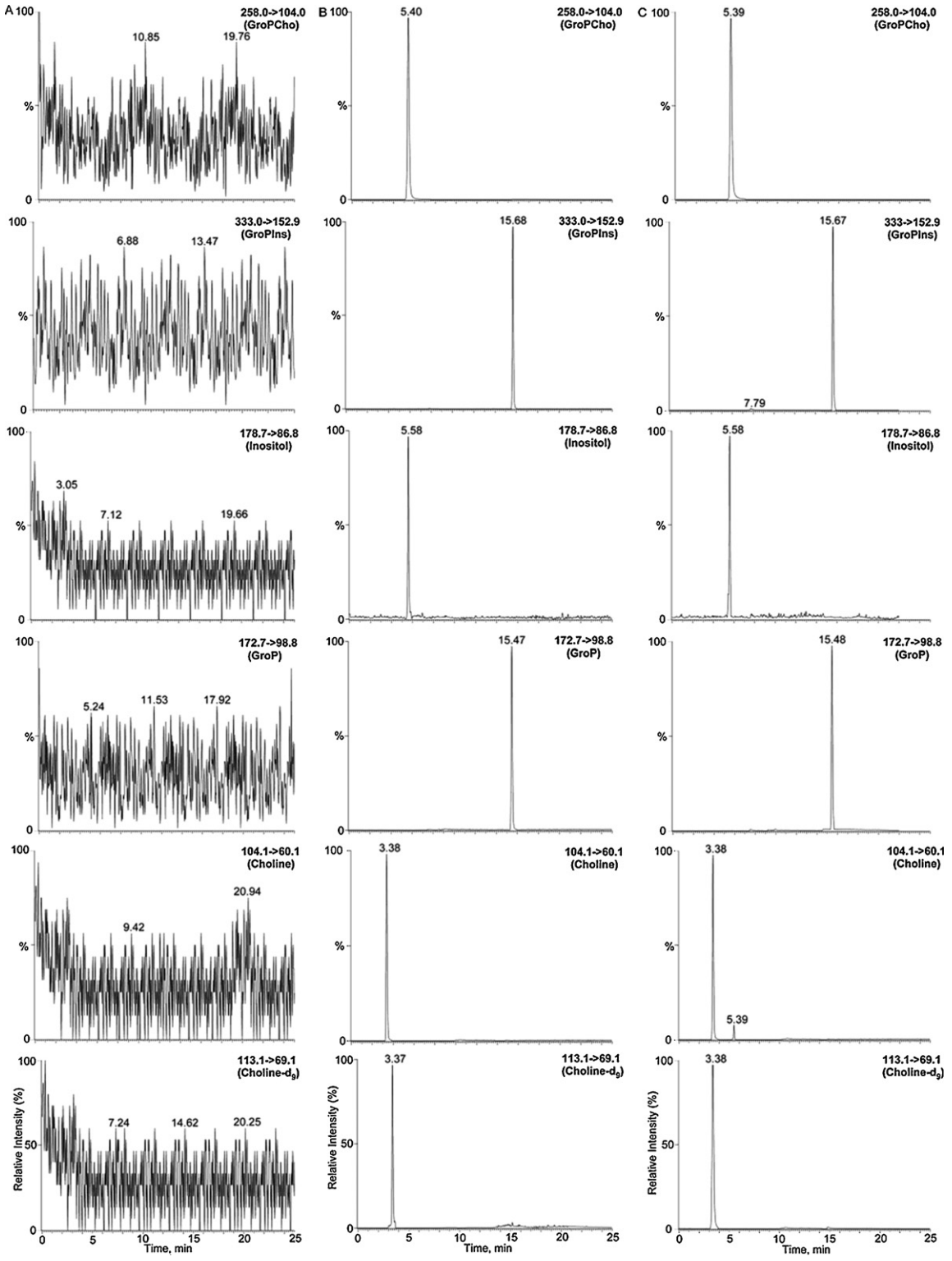

Fig. 3. Representative LC–MS/MS chromatograms of (A) a blank medium (noise baseline), (B) medium sample spiked with analytes and internal standard at 0.5 nM (LLOQ), and (C) medium from a wild type strain (JPV203) spiked with internal standard at 0.5 nM.

the standards spiked in mobile phase (10 mM ammonium acetate in acetonitrile/water, 70:30 (v/v)), were measured to be 95.6%, 97.7%, 98.4%, 95.3%, and 97.0% for GroPIns, GroPCho, GroP, inositol, and choline, respectively (Table 4). The matrix effect value of <100% indicates ionization suppression. The matrix was found to have negligible impact on the ionization, as the variation in signal was less than 10% for all analytes. The absolute matrix effect value of IS at 50 nM in the medium was observed to be 96.7%.

3.3.6. Stability

The stability of QC samples at 2 and 100 nM were determined after the following manipulations: three freeze/thaw cycles, 4-h

storage at room temperature, 30–day storage at −20 °C, and autosampler reinjection after 24 h (Table 5). After 24 h at room temperature there was an acceptable but significant loss of analytes (about 10%). QC samples were stable in different storage conditions, as their assay values were within the acceptable limits of accuracy (±15% RE) and precision (±15% CV).

3.4. Application to a biological analysis

To apply the developed method to a biological system, we monitored the medium of *S. cerevisiae* cultures containing wild type cells and cultures containing mutant strains in which one or more of the phospholipase B genes had been deleted. We first monitored the culture medium prior to the addition of cells in order to get a zero time reading (Fig. 4A) and again after several hours, at which point the cells had reached the stationary phase of growth (Fig. 4B).

Based on prior literature using radiolabeling, we expected to see a decrease in GroPCho levels in strains lacking the *PLB1* gene and a decrease in GroPIns levels in strains lacking the *PLB3* gene, but had no firm estimate of the absolute quantities of these metabolites [20,21,46]. Since we supplemented the medium with inositol and choline, we expected to see a decrease in those metabolites as a function of growth, but did not have an estimate of the extent of uptake by the cells. With regard to GroP, we did not know if this compound would be detected extracellularly and had no expectation regarding its extracellular concentration. As shown in Fig. 4A, the mean concentrations of inositol and choline in blank medium were determined to be 61.8 μM and 15.18 μM. As shown in Fig. 4B, the concentration of inositol decreased in all strains to approximately 22–25 μM. Interestingly, the concentration of choline in the medium decreased to a greater extent for the wild type strain than it did for any of the strains bearing a mutated copy of a *PLB* gene. This finding suggests that either choline transport is diminished in the mutant strains or that the production and subsequent release of choline is exacerbated in the mutant strains. Further studies will be needed in order to reconcile these possibilities. Also interesting is that not only is GroP detected in the medium, but that the level is greater in the wild type strain than in any of the mutant strains. Again, further experiments will be needed to understand the metabolic underpinnings of this result. With regard to GroPCho levels, we observed the most severe decrease in levels in the *plb1*Δ and *plb1-3*Δ mutant as compared to the wild type strain, as expected. Somewhat surprising was that the *plb1*Δ and *plb3*Δ mutants also exhibited a decrease in GroPCho, whereas the published data using radiolabeling saw little or no decrease in that metabolite in the *plb2*Δ and *plb3*Δ strains [20,21,46]. These differences may be due to the limitations associated with radiolabeling with ^{14}C-choline, since only the pool of choline taken up by the cell is labeled and not that derived from de novo synthesis. Also genetic differences between the strain backgrounds and variations in the time of growth between the experiments may account for these differences. With regard to GroPIns levels, we did not see a decrease in the *plb3*Δ mutant strain as expected from the literature [20,21,46]. Therefore, we performed another experiment using only a wild type strain and a *plb3*Δ mutant (Fig. 4C) in which we monitored the level of metabolites at an earlier point in their growth phase, the logarithmic phase of growth, as careful examination of the published reports suggest that those studies were likely performed with logarithmically growing cells. Under those conditions, we observed the expected approximately 50% decrease in GroPIns levels in the *plb3*Δ mutant as compared to wild type.

To summarize the biological application of this newly developed methodology, we were able to confirm the general trends expected for the extracellular levels of GroPIns and GroPCho in wild type and mutant strains. However, much additional information was obtained regarding the absolute levels of these metabolites and the levels of GroP, inositol, and choline in the medium as a function of growth and the absence of particular PLB-encoding genes. Thus, the application of this approach has opened up new avenues of potential research. We expect this method will be of interest to researchers studying phospholipid metabolism in *S. cerevisiae* and to researchers interested in metabolic footprinting as a means of monitoring the metabolic status of any cell type.

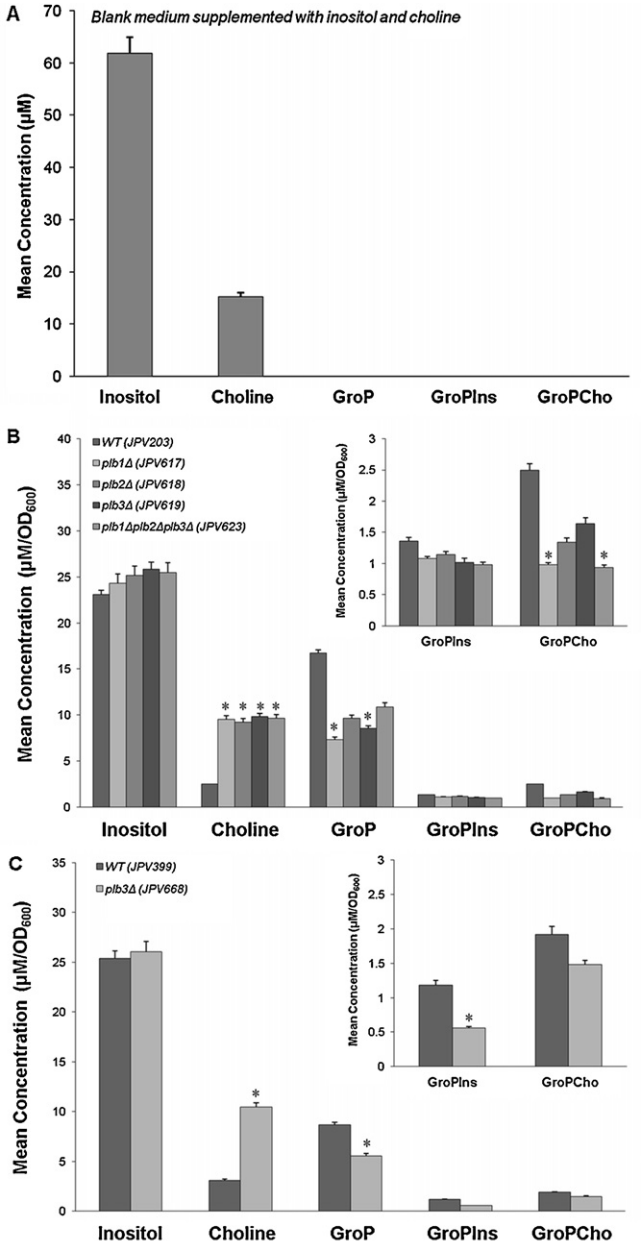

Fig. 4. Determination of lipid-related extracellular metabolites of *S. cerevisiae* in wild cells and in response to deletion of *PLB1*, *PLB2*, and *PLB3* genes encoding for phospholipases B. (A) Blank medium supplemented with 75 μM inositol, and 20 μM choline was analyzed at time 0. Strains grown in medium were harvested at either (B) stationary phase after approximately 24 h of growth for the PLB activity assays (C) log phase after 10–12 h of growth. Data of (B) and (C) presented as concentration (μM) as a function of cell growth (OD_{600}). The insets in (B) and (C) showed the mean concentrations of GroPIns and GroPCho in detail. A 0.5 nM internal standard was used. Data represent the average of three independent experiments per group ± SE. A Student's *t*-test was performed for all analytes comparing each mutant to the wild type with $n = 3$, $^{*}p < 0.05$.

Table 5
Stability of QC samples ($n = 6$).

Analyte	Storage condition	LQC			HQC		
		Mean (nM)	PRE[a] (CV%)	ACC[b] (RE%)	Mean (nM)	PRE (CV%)	ACC (RE%)
GroPIns	FT-3	1.97	2.72	−1.54	96.28	4.67	−3.85
	Short-T	1.99	4.63	−0.58	97.52	5.05	−2.54
	Long-T	2.02	3.57	−1.45	95.05	3.52	−5.62
	Auto-R	1.85	4.48	−7.56	89.94	4.34	−10.11
GroPCho	FT-3	1.96	5.12	−2.81	97.77	2.78	−2.36
	Short-T	1.98	4.80	−1.70	99.56	3.41	−0.58
	Long-T	1.94	4.27	−3.35	93.23	2.90	−6.84
	Auto-R	1.88	3.92	−6.12	91.02	5.33	−9.42
GroP	FT-3	1.97	3.16	−0.15	100.43	4.01	0.49
	Short-T	2.01	2.57	0.54	97.22	4.26	−2.84
	Long-T	1.90	4.43	−5.03	96.39	5.53	−3.76
	Auto-R	1.89	3.87	−5.56	88.54	3.11	−11.52
Inositol	FT-3	1.96	2.64	−2.68	94.60	2.46	−5.44
	Short-T	2.02	4.39	1.32	97.91	5.18	−2.16
	Long-T	1.98	3.61	−1.60	95.13	3.62	−4.92
	Auto-R	1.84	4.74	−7.25	90.67	4.54	−9.47
Choline	FT-3	2.03	5.89	1.59	97.92	4.36	−2.18
	Short-T	1.95	3.72	−2.52	99.36	2.91	−0.72
	Long-T	1.91	3.24	−4.52	89.63	3.83	−10.45
	Auto-R	1.87	2.67	−6.58	90.42	4.06	−9.64

Abbreviations: FT-3, three freeze/thaw cycles; short-T, 4-h storage at room temperature; long-T, 30-day storage at −20 °C; Auto-R, autosampler reinjection after 24 h at room temperature.

[a] [Standard deviation/mean concentration measured] × 100.

[b] [(Mean concentration measured − nominal concentration)/nominal concentration] × 100.

4. Conclusions

We have successfully developed and validated an efficient analytical method based on HILIC–MS/MS for the quantification of lipid-related extracellular metabolites in *S. cerevisiae*. In comparison with traditional radiolabeling methods and other LC–MS/MS methods, our approach is simple, robust, and well suited to metabolomic studies.

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References

[1] S.G. Oliver, M.K. Winson, D.B. Kell, F. Baganz, Trends Biotechnol. 16 (1998) 373.
[2] R.J. Bino, R.D. Hall, O. Fiehn, J. Kopka, K. Saito, J. Draper, B.J. Nikolau, P. Mendes, U. Roessner-Tunali, M.H. Beale, R.N. Trethewey, B.M. Lange, E.S. Wurtele, L.W. Sumner, Trends Plant Sci. 9 (2004) 418.
[3] D.B. Kell, M. Brown, H.M. Davey, W.B. Dunn, I. Spasic, S.G. Oliver, Nat. Rev. Microbiol. 3 (2005) 557.
[4] M. Mashego, K. Rumbold, M. De Mey, E. Vandamme, W. Soetaert, J. Heijnen, Biotechnol. Lett. 29 (2007) 1.
[5] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass Spectrom. Rev. 26 (2007) 51.
[6] S. Schiesel, M. Lämmerhofer, W. Lindner, Anal. Bioanal. Chem. 396 (2010) 1655.
[7] S.G. Villas-Bôas, J. Højer-Pedersen, M. Åakesson, J. Smedsgaard, J. Nielsen, Yeast 22 (2005) 1155.
[8] S.G. Villas-Bôas, S. Noel, G.A. Lane, G. Attwood, A. Cookson, Anal. Biochem. 349 (2006) 297.
[9] S.G. Villas-Bôas, J.F. Moxley, M. Akesson, G. Stephanopoulos, J. Nielsen, Biochem. J. 388 (2005) 669.
[10] J. Allen, H.M. Davey, D. Broadhurst, J.K. Heald, J.J. Rowland, S.G. Oliver, D.B. Kell, Nat. Biotechnol. 21 (2003) 692.
[11] J. Allen, H.M. Davey, D. Broadhurst, J.J. Rowland, S.G. Oliver, D.B. Kell, Appl. Environ. Microbiol. 70 (2004) 6157.
[12] S. Mas, S.G. Villas-Boas, M.E. Hansen, M. Akesson, J. Nielsen, Biotechnol. Bioeng. 96 (2007) 1014.
[13] N.N. Kaderbhai, D.I. Broadhurst, D.I. Ellis, R. Goodacre, D.B. Kell, Comp. Funct. Genomics 4 (2003) 376.
[14] M. Bedair, L.W. Sumner, TrAC Trends Anal. Chem. 27 (2008) 238.
[15] M. Brown, W.B. Dunn, D.I. Ellis, R. Goodacre, J. Handl, J.D. Knowles, S. O'Hagan, I. Spasic, D.B. Kell, Metabolomics 1 (2005) 39.
[16] O. Fiehn, TrAC Trends Anal. Chem. 27 (2008) 261.
[17] R. Goodacre, S. Vaidyanathan, W.B. Dunn, G.G. Harrigan, D.B. Kell, Trends Biotechnol. 22 (2004) 245.
[18] W. Weckwerth (Ed.), Metabolomics. Methods and Protocols, Humana Press, Seacaucus, NJ, 2007.
[19] R.C. Dickson, J. Lipid Res. 49 (2008) 909.
[20] K.S. Lee, J.L. Patton, M. Fido, L.K. Hines, S.D. Kohlwein, F. Paltauf, S.A. Henry, D.E. Levin, J. Biol. Chem. 269 (1994) 19725.
[21] O. Merkel, M. Fido, J.A. Mayr, H. Pruger, F. Raab, G. Zandonella, S.D. Kohlwein, F. Paltauf, J. Biol. Chem. 274 (1999) 28121.
[22] J.L. Patton-Vogt, S.A. Henry, Genetics 149 (1998) 1707.
[23] G.M. Carman, S.A. Henry, J. Biol. Chem. 282 (2007) 37293.
[24] G.M. Carman, G.-S. Han, Annu. Rev. Biochem. 80 (2011) 859.
[25] P.-V. Jana, Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1771 (2007) 337.
[26] S.R. Dowd, M.E. Bier, J.L. Patton-Vogt, J. Biol. Chem. 276 (2001) 3756.
[27] J.L. Patton, L. Pessoa-Brandao, S.A. Henry, J. Bacteriol. 177 (1995) 3379.
[28] J.L. Patton-Vogt, P. Griac, A. Sreenivas, V. Bruno, S. Dowd, M.J. Swede, S.A. Henry, J. Biol. Chem. 272 (1997) 20873.
[29] J.P. Fernández-Murray, C.R. McMaster, J. Biol. Chem. 280 (2005) 38290.
[30] S.J. Cook, M.J. Wakelam, Biochem. J. 263 (1989) 581.
[31] M.E. Koch-Kallnbach, H. Diringer, Hoppe Seylers Z Physiol. Chem. 358 (1977) 367.
[32] L.K. Dragani, C.P. Berrie, D. Corda, D. Rotilio, J. Chromatogr. B 802 (2004) 283.
[33] F. Kopp, T. Komatsu, D.K. Nomura, S.A. Trauger, J.R. Thomas, G. Siuzdak, G.M. Simon, B.F. Cravatt, Chem. Biol. 17 (2010) 831.
[34] M. Sato, S. Dhut, T. Toda, Yeast 22 (2005) 583.
[35] U. Güldener, S. Heck, T. Fiedler, J. Beinhauer, J.H. Hegemann, Nucleic Acids Res. 24 (1996) 2519.
[36] S.U. Bajad, W. Lu, E.H. Kimball, J. Yuan, C. Peterson, J.D. Rabinowitz, J. Chromatogr. A 1125 (2006) 76.
[37] B. Barroso, R. Bischoff, J. Chromatogr. B 814 (2005) 21.
[38] L. Coulier, R. Bas, S. Jespersen, E. Verheij, M.J. van der Werf, T. Hankemeier, Anal. Chem. 78 (2006) 6573.
[39] T. Sun, S. Pawlowski, M.E. Johnson, Anal. Chem. 83 (2011) 6628.
[40] S. Cubbon, T. Bradbury, J. Wilson, J. Thomas-Oates, Anal. Chem. 79 (2007) 8911.
[41] T. Kind, V. Tolstikov, O. Fiehn, R.H. Weiss, Anal. Biochem. 363 (2007) 185.
[42] X. Pan, D.S. Yuan, D. Xiang, X. Wang, S. Sookhai-Mahadeo, J.S. Bader, P. Hieter, F. Spencer, J.D. Boeke, Mol. Cell 16 (2004) 487.
[43] M. Piraud, C. Vianey-Saban, C. Bourdin, C. Acquaviva-Bourdain, S. Boyer, C. Elfakir, D. Bouchu, Rapid Commun. Mass Spectrom. 19 (2005) 3287.
[44] H. Schlichtherle-Cerny, M. Affolter, C. Cerny, Anal. Chem. 75 (2003) 2349.
[45] V.V. Tolstikov, A. Lommen, K. Nakanishi, N. Tanaka, O. Fiehn, Anal. Chem. 75 (2003) 6737.
[46] O. Merkel, O.V. Oskolkova, F. Raab, R. El-Toukhy, F. Paltauf, Biochem. J. 387 (2005) 489.