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Yeast Functional Analysis Report

Mass spectrometry-based profiling of phospholipids and sphingolipids in extracts from *Saccharomyces cerevisiae*

Xue Li Guan and Markus R. Wenk*
Department of Biochemistry and Department of Biological Sciences, National University of Singapore, Singapore 117597

**Correspondence to:*
Markus R. Wenk, National University of Singapore, Yong Loo Lin School of Medicine, Department of Biochemistry, 8 Medical Drive, Block MD7, Singapore 117597.
E-mail: bchmrw@nus.edu.sg

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Abstract

Lipids are rapidly moving to centre stage in many fields of biological sciences. Lipidomics, the systems-level scale analysis of lipids and their interacting factors, is thus an emerging field which holds great promise for drug and biomarker discovery. Here we present a mass spectrometry-based approach for profiling of polar lipids, in particular phospholipids and sphingolipids, in *Saccharomyces cerevisiae*. The first step includes semi-quantitative surveys of lipids in an untargeted fashion, which is particularly powerful for detection of changes that cannot easily be anticipated. This leads to the identification of ions with increased or decreased signal intensities. Comprehensive theoretical calculation of the masses of yeast phospholipid and sphingolipid molecular species, based on fatty acyl and headgroup heterogeneity, is next used to tentatively assign ions of interest. Subsequent targeted analysis using tandem mass spectrometry allows for characterization and quantification of phospholipids and sphingolipids. Given the high degree of conservation in pathways of lipid metabolism between different organisms, it can be expected that this method will lead to the discovery of novel enzymatic activities and modulators of known ones, particularly when used in combination with genetic and chemogenetic libraries and screens. We validated the method using the EUROSCARF library of non-essential deletion mutants. Mutants of *SCS7*, a lipid hydroxylase, and *SLC1*, a putative acyl transferase with unknown substrate specificity, were profiled for their phospholipid and sphingolipid content. The observed changes in lipid profiles are consistent with previous observations and extend our knowledge on *in vivo* substrate use under permissive growth conditions.

Keywords: **mass spectrometry; *S. cerevisiae*; phospholipid; sphingolipid; lipidomics**

Introduction

Lipids are small molecules which play important roles in physiology and disease. They are building blocks of cellular membranes, precursors for signalling molecules and they serve as storage for chemical energy in highly reduced form. At the moment the field of lipid research is undergoing revolutionary developments, driven by (a) technological advances for lipid detection, and (b) insights from recent functional characterization of lipid enzymes in model systems.

Studies in *S. cerevisiae* have played a particularly important role in the advancements of our knowledge of lipid function. As a result, much is known about the pathways, and their regulation, of (phospho)lipid biosynthesis in yeast (Homann *et al*., 1987; Carman and Henry, 1989; Kohlwein *et al*., 1996; Daum *et al*., 1998; Vance, 2003). In addition, and more recently, elegant experiments using combined genetic, biochemical and functional approaches have shed light on the role of phosphoinositides in secretion (Odorizzi *et al*., 2000; Huijbregts *et al*., 2000; Simonsen *et al*., 2001), the role of sterols in endocytic events (D'Hondt *et al*., 2000; Heese-Peck *et al*., 2002) and triglyceride synthesis for storage of lipids in intracellular deposits (Zweytick *et al*., 2000; Mullner and Daum, 2004).

While many of these studies were aimed at investigating physiological processes (such as membrane trafficking) in yeast, it is clear that they also advanced tremendously our understanding of lipid metabolism in other organisms, including mammals. It is important to note that many pathways of lipid biosynthesis and metabolism are well conserved between species ranging from yeast to worm to mouse and human. There are also, however, a number of notable differences in yeast lipids compared to mammalian counterparts. For example, *S. cerevisiae* synthesizes ergosterol as its major sterol lipid, as opposed to cholesterol in mammalian cells (Zinser *et al*., 1993; Munn *et al*., 1999); yeast sphingolipids contain inositol mannosyl residues, rather than glucosyl sugars found in complex glycolipids in humans (Daum *et al*., 1998).

Traditional lipid analysis, such as thin-layer and gas chromatography, is hampered by limited sensitivity, selectivity and resolution. Gas chromatography, for example, is a powerful method for determination of fatty acyl heterogeneity but it requires derivatization and pertinent standards. In addition, information on the origin of the fatty acyl moiety is generally lost. Metabolic labelling using lipid precursors (such as radiolabelled inositol, serine or fatty acyls) have been widely used to (selectively) label certain classes of lipids, which are then typically separated using thin-layer chromatography (TLC) and visualized by autoradiography. While these approaches are generally easy to use and do not require specialized equipment, they only deliver mass levels of lipids under conditions of steady-state incorporation of the label (which sometimes is difficult to achieve technically). In addition, TLC separation is generally of low resolution.

Electrospray ionization mass spectrometry (ESI–MS) with minimal or no sample preseparation has been used for analysis of lipids with great success (Faergeman *et al*., 2004; Forrester *et al*., 2004; Wenk, 2005; Han and Gross, 2005). The major advantage of this method is its capability to detect large numbers of individual lipid ions (currently in the order of hundreds to a few thousands), with different chemistries, in complex mixtures and a single experiment (Hughey *et al*., 2002; Mougous *et al*., 2002; Han and Gross, 2005).

Here we describe such a mass spectrometry-based approach to profile lipids from minimally processed organic extracts of *S. cerevisiae*. The method is based on (a) high-resolution analysis for detection of global changes ('untargeted profiling'), followed by (b) characterization and quantification of changes of interest using tandem mass spectrometry (MS–MS), MS^3 (MS-MS-MS), and multiple reaction monitoring (MRM). We furthermore present comprehensive theoretical calculations of molecular masses for yeast phospholipids and sphingolipids, of which a large number can be measured experimentally in a single extract using the present method. This method will be particularly useful for large-scale studies which may include screening of (chemo)genetic libraries for discovery of novel enzymatic entities.

Materials and methods

Strains, media and culture conditions

The strains used in this study (Table 1) are obtained from the European *S*accharoymces *c*erevisiae Archive for Functional Analysis (EUROSCARF) library. BY4741 is a wild-type strain, while Δ*slc1* and Δ*scs7* are deletion mutants for genes encoding a putative acyltransferase (*SLC1*) and sphingolipid α-hydroxylase (*SCS7*), respectively. Cells were kept on YPD plates and inoculated in YPD medium (1% yeast extract, 2% Bacto-peptone and 2% glucose). The cells were then diluted into 80 ml fresh medium to an optical density (OD) at 600 nm (OD_{600}) of 0.075 and grown to logarithmic phase ($OD_{600} \cong 0.65–0.85$). All steps were carried out at 30 °C in a rotary shaker (250 ml flasks). At least four different colonies of each strain were used for independent analysis.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
BY4741 (wild type)	MATa; his3D1; leu2D0; met15D0; ura3d0	EUROSCARF
Δ*scs7*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YMR272c::kanMX4	EUROSCARF
Δ*slc1*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YDL052c::kanMX4	EUROSCARF

Lipid standards

Synthetic lipids with fatty acyl compositions that are naturally of low abundance in *S. cerevisiae* were used as internal standards. Phosphatidic acid with a C17 chain (C17-PA), C19-ceramide (C19-CER), C8 glucosylceramide (C8-GC) and liver phosphatidylinositol (PI) were obtained from Avanti Polar Lipids (Alabaster, AL). The internal standards were solubilized in chloroform at a stock concentration of 10 μg/μl.

Lipid extraction

25 OD_{600} equivalents of yeast cells were harvested by centrifugation at 4000 r.p.m. for 5 min and washed twice with deionized water. Lipids were extracted by a method described by Angus and Lester (1972), with slight modifications. Briefly, the cells were resuspended in 2 ml 95% ethanol : water : diethyl ether : pyridine : ammonium hydroxide (15 : 15 : 5 : 1 : 0.018). 1 μg C19-Cer, 0.5 μg C8-GC, 5 μg liver PI and 2.5 μg C17-PA were added as internal standards. The cells were broken by glass beads vortexing (twice for 1 min each) and incubated for 20 min at 60 °C. Debris was pelleted by centrifugation and the supernatant was transferred to a fresh tube. The pellet was re-extracted once more using the same procedure. The pooled supernatants were divided into equal aliquots and dried under a stream of nitrogen. One aliquot was used for total lipid analysis (mainly phospholipids) and the other for sphingolipid analysis (see also Figure 1).

For total lipid extraction, the dried lipid film was desalted by butanol extraction. A fraction enriched in sphingolipids was obtained by mild alkaline hydrolysis, which degrades ester linkages found in many glycerophospholipids (Brockerhoff, 1963). To achieve this, the dried lipid films were resuspended in 400 μl chloroform : methanol : water (16 : 16 : 5, v/v/v). Glycerophospholipids were deacylated by 400 μl 0.2 N NaOH and incubation at 30 °C for 45 min. 400 μl 0.5 M EDTA was added and the samples were neutralized with 1 N acetic acid. Lipids were then extracted with chloroform and dried under nitrogen.

Mass spectrometry

Electrospray ionization mass spectrometry (ESI–MS) was performed using a Waters Micromass Q-Tof Micro (Waters Corp., Milford, MA) mass spectrometer. The dried lipid extracts were reconstituted in 2 ml chloroform : methanol (1 : 1, v/v). Typically, 2 μl sample was injected for mass spectrometry analysis. The capillary voltage and sample cone voltage were maintained at 3.0 kV and 50 V, respectively. The source temperature was 80 °C and the nano-flow gas pressure was 0.7 bar. The mass spectrum was acquired from a mass-to-charge ratio (m/z) of 400–1400 in the negative ion mode, with an acquisition time of 3 min, and the scan duration was 1 s. The HPLC system, consisting of a Waters CapLC Autosampler and a Waters CapLC Pump, was used to provide the mobile phase and to inject samples. Chloroform : methanol (1 : 1, v/v) at a flow rate of 15 μl/min was used as the mobile phase. Individual molecular species were identified using tandem mass spectrometry and in general, the collision energy used was in the range 25–80 eV (see also Figure 5).

Tandem mass spectrometry for characterization/identification as well as quantification of lipid

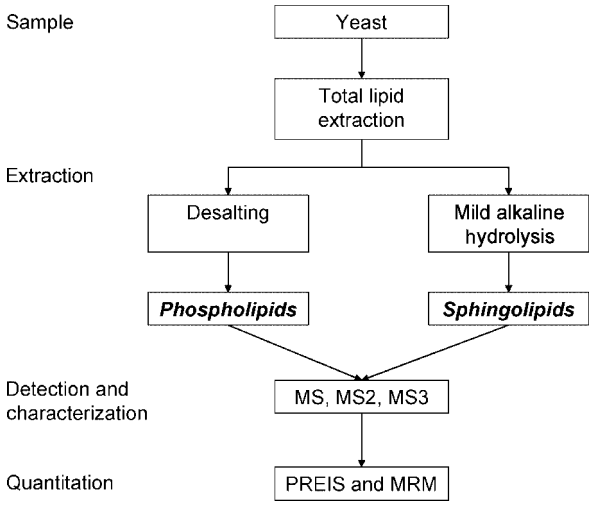


Figure 1. Workflow of the method. A cell pellet from 25 OD units of yeast is extracted using ethanol : water : diethyl ether : pyridine : ammonium hydroxide ('total lipid extract'). This fraction contains both phospholipids and sphingolipids. The fraction is then split and processed for analysis of major phospholipids (which includes a 'desalting' step) and sphingolipids (which resist 'mild alkaline hydrolysis'). Single-stage mass spectrometry (MS) is used to profile the extracts in a non-targeted fashion. Tandem mass spectrometry (MS–MS) and MS^3 is employed to further characterize and quantify lipids of interest (precursor ion scanning, PREIS and multiple reaction monitoring, MRM)

molecular species was performed using precursor ion scanning (PREIS) and multiple reaction monitoring (MRM), respectively, with an Applied Biosystems 4000 Q-Trap mass spectrometer (Applied Biosystems, Foster City, CA) (see also Figures 1, 5). Samples were directly infused using a Harvard syringe pump at a flow rate of 10 µl/min. In MRM experiments, the first quadrupole, Q1, was set to pass the precursor ion of interest to the collision cell, Q2, where it underwent collision-induced dissociation, and next the third quadruple, Q3, which was set to pass the structure-specific product ion characteristic of the lipid of interest (Figure 5). Each individual ion dissociation pathway was optimized with regard to collision energy to minimize variations in relative ion abundance due to differences in rates of dissociation. Lipid concentrations were calculated relative to the relevant internal standards.

Data analysis

Data was acquired using MassLynx 4.0 (Waters Corp., Milford, MA). The plain text files obtained from MassLynx were loaded into Matlab (The MathWorks Inc., Natick, MA) for processing (Guan *et al*., in press). Briefly, correlation-optimized warping (COW) (Nielsen *et al*., 1998) was used as a pre-processing method in order to obtain precise alignment of normalized MS spectra from replicate samples. For averaging of spectra from replicate independent samples, each ion intensity was normalized to the sum of all ion intensities and the normalized data of each replicate was warped against a reference set. After aligning the peaks, the intensity values of individual m/z were then averaged to obtain one mean spectrum representative of the replicates. To compare between different experimental conditions, the mean spectrum for one experimental condition (e.g. mutant) was warped against the mean spectrum for the control condition (wild-type). After alignment, relative differences in the lipid compositions of the mixture can be computed by calculating the logarithm (log10) of the ratio of ion intensities relative to control samples.

Statistical analysis

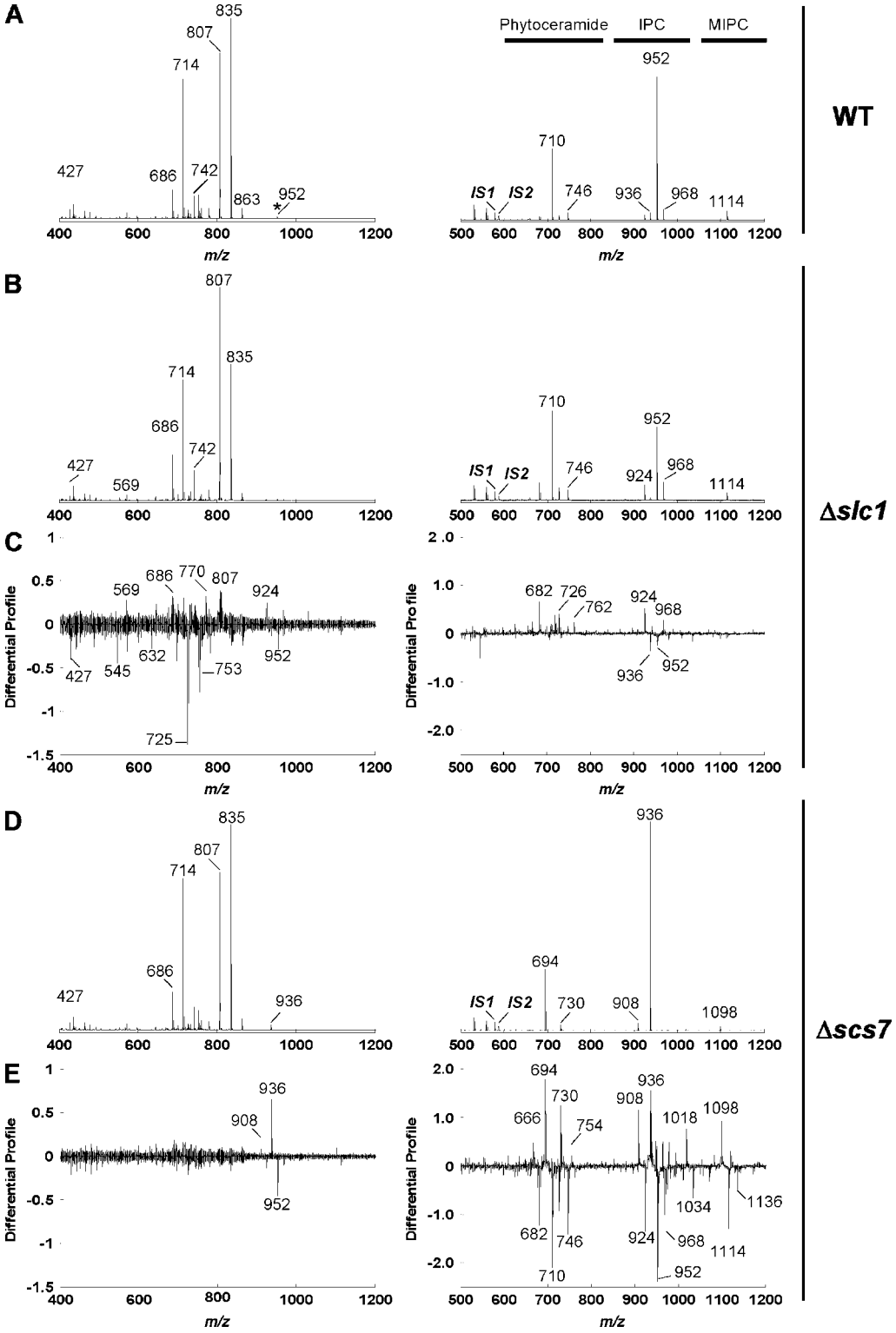
Comparison of the wild-type and mutant strains was performed using the mean of at least four independent biological replicates ± standard error of the mean (SEM) from individual samples. Statistical significance between wild-type and mutant yeast strains was determined using Student's t-test.

Results

The main classes of lipids in *S. cerevisiae*, as in most other eukaryotic cells, are glycerophospholipids, sphingolipids, sterols and glycerolipids (such as diacylglycerols and triacylglycerols), with the polar lipids comprising approximately 40% of total lipids and the remaining 60% comprising of neutral lipids (Blagovic *et al*., 2001). The goal of this study was to establish a simple and rapid method that allows profiling of total polar lipids, containing mainly phospholipids and sphingolipids, as schematized in Figure 1, with a focus on sphingolipids. We chose to validate the approach by using mutant strains that have previously been implicated with aberrant (sphingo)lipid metabolism.

Figure 2A shows a typical profile of a total lipid extract obtained from a wild-type yeast strain (Table 1). The most prominent ions represent major phospholipid species that ionize efficiently in negative mode. The major fatty acyls in yeast are C16 and C18 with none, one or two double bonds.

Figure 2. Phospho- and sphingolipid profiling of yeast mutants. Typical phospholipid (left panels) and sphingolipid profiles (right panels) of wild-type (wild-type) yeast (A) and deletion mutants of the *slc1* (B, C) and *scs7* (D, E) genes. Mass spectra from at least four independent samples were averaged for each condition ($n = 4$). Differential lipid profiles, which are ratios of single-stage MS scans plotted as log10 ratios, are used to compare differences in phospholipid and sphingolipid composition between Δ*slc1* and wild-type (C) and Δ*scs7* and wild-type (E). Note that this approach does not require knowledge of the underlying lipid species for a given ion of interest. Instead, it serves as an 'unbiased' screening tool for the discovery of lipids which are present in different amounts between two conditions. Δ*slc*, a putative acyl transferase, leads to differences in phospholipid profiles (C, left panel) while sphingolipids are less affected when compared to wild-type (C, right panel). The opposite is the case for Δ*scs7*, a ceramide hydroxylase, which displays striking differences in the sphingolipids (E, right panel) with only minor alterations in phospholipids (E, left panel). The asterisk in (A) marks a prominent inositol phosphorylceramide (IPC, m/z 952) species whose signal is substantially increased after alkaline hydrolysis



Yeast, unlike mammalian cells, does not synthesize polyunsaturated fatty acids (Schneiter *et al*., 1999; Blagovic *et al*., 2005). The most prominent inositol phosphorylceramide (IPC) yields a weak signal at *m*/*z* 952 under these conditions (Figure 2A, asterisk).

To improve detection of sphingolipid molecular species, we subjected the total extract to mild alkaline hydrolysis. This treatment hydrolyzes acyl bonds (and hence the majority of glycerophospholipids) but leaves amide linkages largely intact. It has been successfully used for measurement of sphingolipids in mammalian cells and tissues (Merrill *et al*., 2005). Figure 2A shows a high resolution Q-Tof scan of such a sphingolipid-enriched fraction (right panel).

The profiling of complex lipid mixtures in such a fashion, i.e. using single-stage mass spectrometry, serves as a powerful initial screen when different conditions or strains are to be compared. Figure 2B, D shows profiles from Δ*slc1* and Δ*scs7* deletion strains (Table 1). *SLC1* encodes for a putative acyltransferase which has been implicated in the synthesis of phosphatidic acid from lysophosphatidic acid (Dickson *et al*., 1990; Athenstaedt and Daum, 1997). It was originally discovered in a screen for bypass mutants that regain the ability to grow in the absence of sphingolipid biosynthesis. Indeed, *slc1-1* is a suppressor of *lcb1*, which catalyses the committed step of sphingolipid biosynthesis. These original experiments, due to the set-up of the genetic screens, were mainly carried out in the absence of long chain bases (LCB) in the growth medium. Under these conditions, *slc1-1* leads to the generation of unusual inositol-containing glycerophospholipids (Lester *et al*., 1993).

Here we show that under permissive growth conditions, i.e. complete medium rather than media devoid of LCB, the phospholipids resemble largely those found in the wild-type strain (Figure 2B, left panel). A differential profile, which displays differences in ion response between the Δ*slc1* and wild-type conditions, is shown in Figure 2C (left panel). The major differences lie in ions at *m*/*z* 725 and 753, which, based on the mass, can tentatively be assigned to phosphatidylinositols (PI) with a total fatty acyl carbon number of 26 and 0 double bonds (26 : 0-PI) as well as 28 : 0-PI, i.e. PI species with unusually short and saturated fatty acyls. Indeed, a precursor scan for *m*/*z* 241, which is an indicator fragment of inositol-containing lipids, reveals a PI profile which is devoid of these short chain species in the deletion mutant (Figure 3). Interestingly, such short chain PIs have previously been shown to be enriched at the plasma membrane (Schneiter *et al*., 1999).

We next analysed the sphingolipids of Δ*slc1* using the alkali-treated fraction. Major ions correspond in *m*/*z* to those of the wild-type, albeit at altered levels. Ions at *m*/*z* 682, 924, 936 and 952 correspond to t18 : 0/24 : 0 phytoceramide-C, t18 : 0/24 : 0 IPC-C, t18 : 0/26 : 0 IPC-B and t18 : 0/26 : 0 IPC-C, respectively (Supplementary material can be found at **www.lipidprofiles.com/main**). It should be noted that sphingolipids also enter the gas phase efficiently in positive ionization mode. In order to further characterize and identify changes of interest, we used tandem mass spectrometry and collision-induced dissociation (Figure 4 and data not shown). The mannosylated inositol phosphorylceramides (MIPCs) in yeast are lipids with considerable structural complexity. Figure 4A shows the product ion spectra of *m*/*z* 1116 in positive mode (equivalent to *m*/*z* 1114 in negative mode). Two major fragments were observed with *m*/*z* 954 and 694. The daughter ion with *m*/*z* 954 corresponds to the *m*/*z* of 26 : 0 inositol phosphorylceramides (IPC-C). Furthermore, it is equivalent to a loss of 162 mass units from the parent ion, suggesting the loss of a sugar group from MIPC-C. Tandem mass spectrometry of ceramides, and glycosylated derivatives, yield fragments which have to be further characterized for unambiguous identification. Hyphenated MS is a powerful tool for such analysis and Figure 4B shows MS^3 of the most prominent product ion, *m*/*z* 694, derived from collision-induced dissociation of ion of *m*/*z* 1116. MS^3 of *m*/*z* 694 forms dehydration products of phytoceramide as well as (dehydration products) of the t18 : 0 sphingoid bases, thus supporting *m*/*z* 1116 to originate from a C26 MIPC-C with a t18 : 0 sphingoid base (Figure 5). In conclusion, these results indicate that disruption of the *SLC1* gene leads to moderate (yet highly specific) changes in molecular species in phospholipids as well as sphingolipids, most notably complete elimination of very short chain PIs and a concomitant increase in short chain sphingolipids, phytoceramide and IPC.

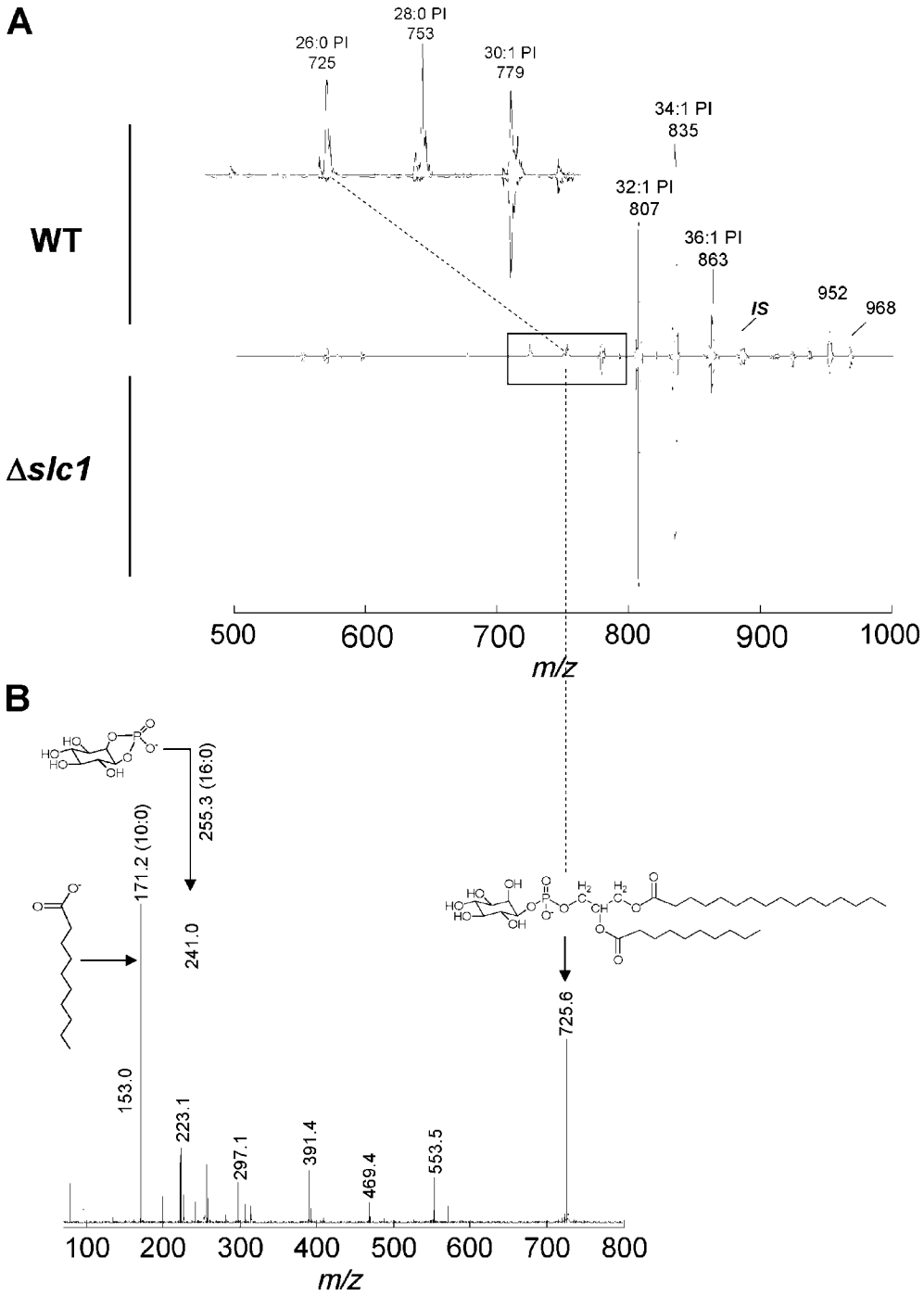


Figure 3. Molecular species of phosphatidylinositol (PI) in Δ*slc1*. (A) Precursor ion scans of *m*/z 241, the mass of dehydrated inositol phosphate [see also (B)] were used to selectively measure PI species in Δ*slc1* (downward profiles) and wild-type (wild-type, upward profiles). Liver PI was spiked into the extracts prior to extraction and used as an internal standard (IS) for quantification. Note the almost complete absence of very short chain PI (26 : 0 PI and 28 : 0 PI) in the mutant strains (inset), which is highly selective. PIs with a total carbon number of 30 and more are present in comparable amounts between Δ*slc1* and wild-type. (B) Tandem mass spectrometry and collision-induced dissociation (CID) of *m*/z 725. Based on theoretical calculation of phospholipid and sphingolipid molecular species, this ion can tentatively be assigned to 26 : 0 PI. This is supported by analysis of the fragment ions, which have the *m*/z characteristic of an inositol (*m*/z 241) containing phospholipid (*m*/z 153), with C10 : 0 (*m*/z 171) and C16 : 0 (*m*/z 255) as the major fatty acyl chains

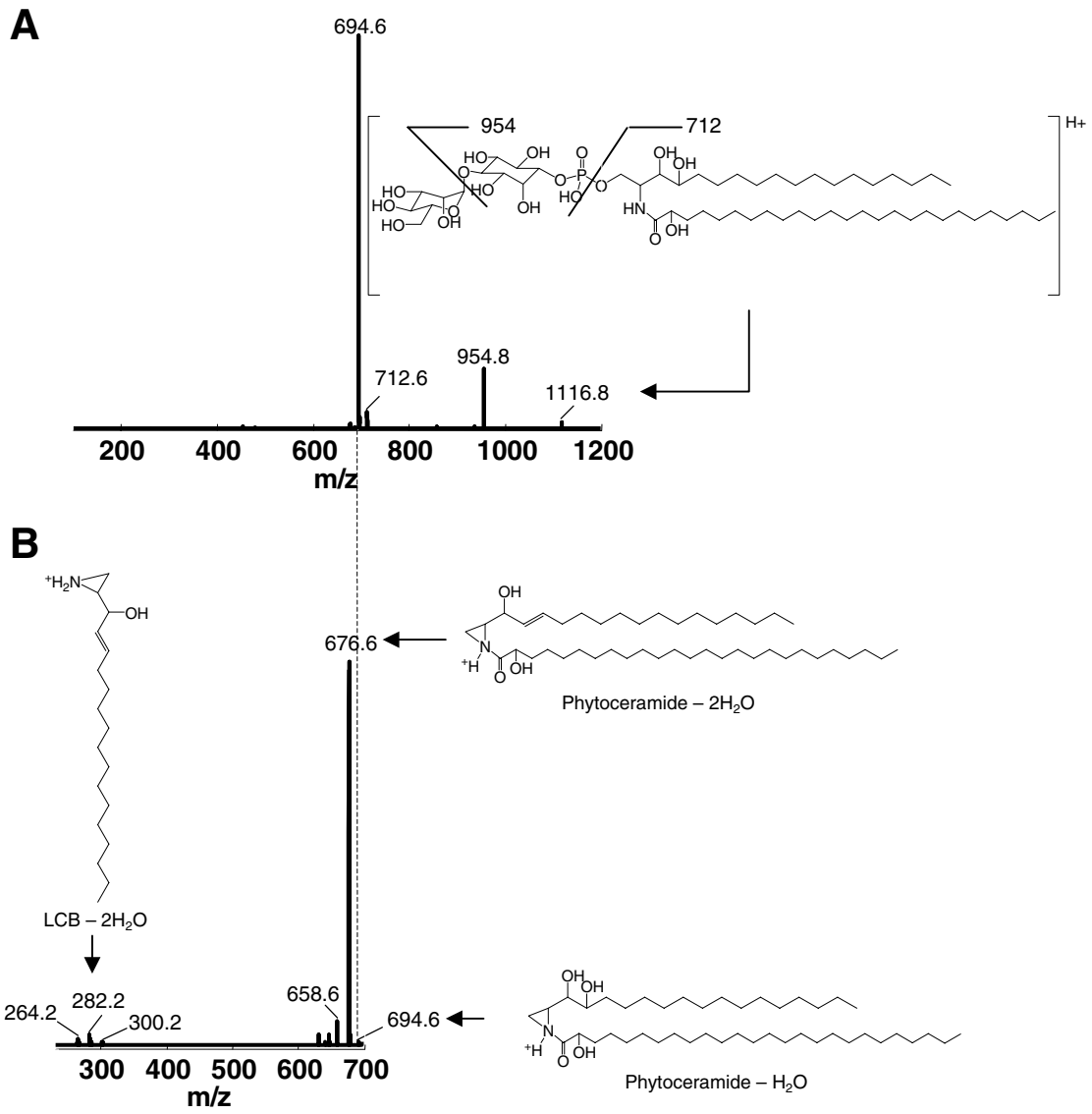
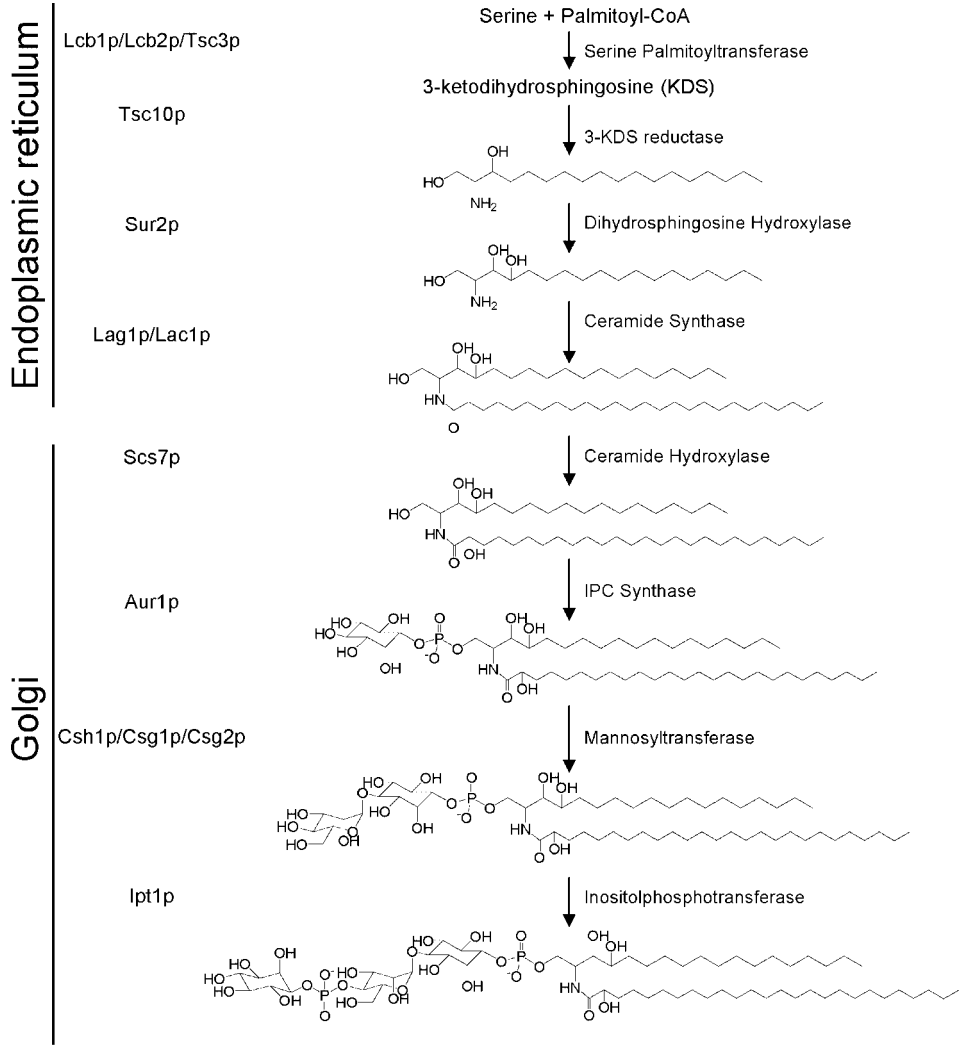


Figure 4. Biochemical characterization of a complex sphingolipid using MS–MS and MS^3. The ion with *m*/*z* 1116.8 can (based on the mass) be tentatively assigned to mannosyl inositol phosphorylceramide (MIPC) of the C-series (t18 : 0/26 : 0, see also Figure 5). Tandem mass spectrometry (positive mode) of *m*/*z* 1116.8 yields a dominant ion at *m*/*z* 694.6, the expected mass for the dehydrated phytoceramide backbone of t18 : 0/26 : 0 MIPC. This is supported experimentally by subsequent MS analysis using MS^3 (on a linear ion trap) of *m*/*z* 694.6, which produces the doubly dehydrated phytoceramide (*m*/*z* 676.6) and fragments corresponding the long chain base (LCB) of the ceramides (e.g. *m*/*z* 282.2)

In contrast, the total lipid profiles of the deletion mutant in the *SCS7* gene displays phospholipids which, in their levels, are almost identical to wild-type conditions (Figure 2D, E, left panels). Ions at *m*/*z* 936 and 952 which are shown in the differential profile stem from abundant sphingolipid species (see also Figure 2D, E, right panels). *SCS7* encodes for sphingolipid α-hydroxylase, an enzyme involved in the generation of monohydroxylated inositol ceramides (Haak *et al*., 1997; Dunn *et al*., 1998). If *SCS7* contributed in a major way to total sphingolipid hydroxylation, one would expect dramatic differences in sphingolipid species. This is indeed revealed experimentally. The levels of



Species	Precursor/ product ion *m/z*	DP (eV)	CE (eV)
Sphingoid Base			
Dihydrosphingosine			
d18:0	302.2/284.2	70	20
d20:0	330.2/312.3	80	20
Phytosphingosine			
t18:0	318.3/282.3	60	25
t20:0	346.3/310.3	100	25
Phytoceramide (Sphingoid base/Fatty acid-Type)			
d18:0/18:0-A	568.6/284.3	60	40
t18:0/18:0-B	584.6/282.3	80	40
t18:0/24:0-C	684.7/282.3	80	45
t18:0/26:0-B	696.7/282.3	80	45
t18:0/26:0-C	712.7/282.3	80	45
t18:0/26:0-D	728.7/282.3	80	50
t20:0/26:0-B	724.7/310.3	100	45
t20:0/26:0-C	740.8/310.3	100	45
t20:0/26:0-D	756.8/310.3	100	50
Inositol Phosphorylceramide (IPC)			
t18:0/24:0-C	926.7/282.3	80	60
t18:0/26:0-B	938.7/282.3	80	65
t18:0/26:0-C	954.7/282.3	100	65
t18:0/26:0-D	970.7/282.3	100	70
t20:0/26:0-B	966.8/310.3	80	70
t20:0/26:0-C	982.8/310.3	80	70
Mannosyl Inositol Phosphorylceramide (MIPC)			
t18:0/26:0-B	1100.8/282.3	100	75
t18:0/26:0-C	1116.8/282.3	100	80
t20:0/26:0-B	1128.8/310.3	100	80
t20:0/26:0-C	1144.8/310.3	100	80
t20:0/26:0-D	1160.8/310.3	100	80
Mannosyl Diinositol Phosphorylceramide (M(IP)2C)			
t18:0/26:0-B	670.2/241.0	-180	-75
t18:0/26:0-C	678.2/241.0	-180	-75
Internal Standard			
d18:1/19:0 Ceramide	580.6/282.3	45	35
d18:1/8:0 Glucosylceramide	588.6/282.3	45	45

Figure 5. Sphingolipid pathway of *S. cerevisiae* and molecular species of lipids covered in this study. Sphingolipid metabolism is compartmentalized in yeast and major activities are found in the endoplasmic reticulum and the Golgi apparatus (far left). Well-characterized mutants which are defective in this pathways are indicated (e.g. Lcb, Tsc10, Sur2, etc.) and aligned with representative molecular structures of sphingolipids. The adjoining table on the right summarizes the precursor/product pairs, declustering potential (DP) and collision energies (CE) used for multiple reaction monitoring (MRM) quantification of yeast sphingolipids. Note that molecular species represented by the structures are initialized in the table. Results obtained using this method for two yeast strains, Δ*slc1* and Δ*scs7* are shown in Figure 6

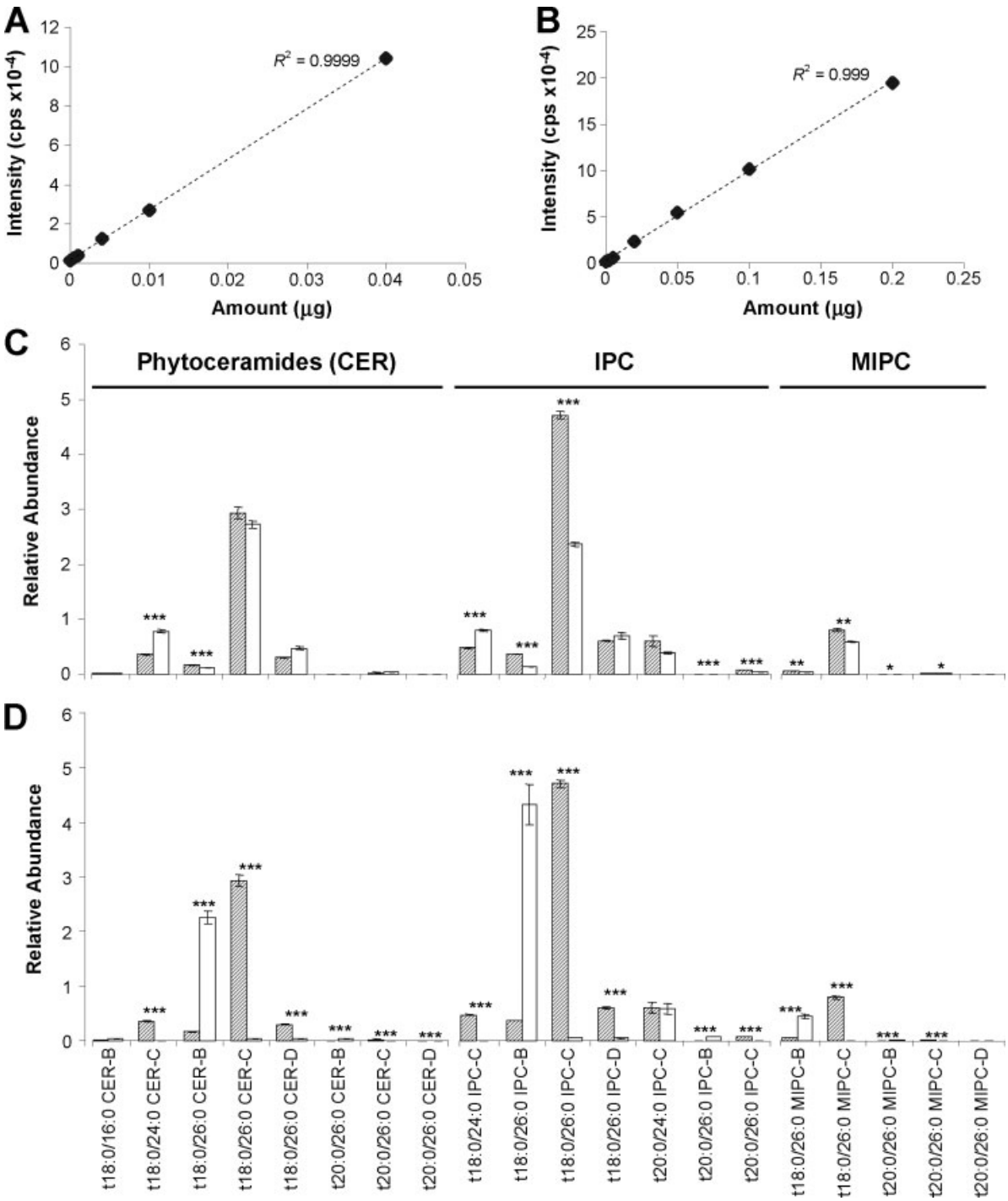


Figure 6. Sphingolipid levels of Δ*slc1* and Δ*scs7* relative to a wild-type strain using multiple reaction monitoring quantification. (A, B) The ion response (in MRM quantification mode, see also Figure 5) for increasing amounts of t18:0 phytosphingosine and t18:0/18:0 phytoceramide standards, respectively. Comparison of sphingolipid molecular species in wild-type (hatched bars), Δ*slc1* (C, open bars), and Δ*scs7* (D, open bars). Data are presented as means ± SEM of four independent experiments. Statistical significance between wild-type and mutant strains was determined using Student's t-test: $^{*}p < 0.01$; $^{**}p < 0.005$; $^{***}p < 0.001$

hydroxylated ceramides and complex ceramides are drastically reduced in the mutant (Figure 2D, E, right panels). This is mirrored with almost identical concomitant increases in the 'B' series for ceramide species, i.e. those ceramides which do not carry a hydroxyl group on the fatty-acyl chain (Figures 5, 6D).

The above results demonstrate the power of comparative single-stage analysis of complex lipid mixtures. The mass of phospholipids and sphingolipids, including their mannosylated derivatives, can be calculated based on chemical composition (Marsh, 1990; Wenk and De Camilli, 2005). This leads to a large array of theoretical lipid species, of which 5–10% are found in typical yeast extracts. Such a combined experimental and theoretical approach is an excellent tool for the discovery of unexpected changes in lipid levels and lipid species. It also sets the stage for subsequent targeted quantification of lipids of interest.

Multiple reaction monitoring (MRM) is a highly selective and sensitive (although biased) method for the quantification of small molecules in complex mixtures. It requires information on specific fragments as well as pertinent internal standards. The method is based on monitoring of specific parent ion–fragment ion pairs, which are filtered in the two quadrupoles of a tandem mass spectrometer. Figure 5 contains the MRM pairs of yeast sphingolipids covered in this study. We next used MRM to quantify changes in sphingolipids in Δ*slc1* and Δ*scs7* (Figure 6). Note the generally good agreement between this full quantitative analysis and the semi-quantitative pre-screening shown in Figure 2.

Discussion

We describe here a novel mass spectrometry-based approach for the screening of lipid metabolites in the yeast *S. cerevisiae*, with a focus on phospholipids and sphingolipids. Using a rather simple extraction protocol, it is possible to obtain semi-quantitative information of overall changes in lipid levels (Figure 2) as well as quantitative levels of a large number of lipids in the sphingolipid pathway, including mannosylated inositol ceramides (Figure 6). While full automation is difficult to achieve for liquid/liquid extraction, it is now feasible to analyse ~100 strains/day of mass spectrometry time. Extraction of yeast cells typically requires 6 h, including incubation and drying steps. Thus, it will be possible to screen libraries of mutant yeast strains for discovery of mutants with defects in certain aspects of phospholipid and sphingolipid metabolism.

A major advantage of this method is that it does not require metabolic labelling (e.g. use of radioactive precursors) and sample preparation, and clean-up is minimal. The method is robust and directly measures mass levels of these lipids. It can thus be used as a discovery tool in many applications, ranging from experiments which address basic molecular mechanisms of membrane traffic (Odorizzi *et al*., 2000; Huijbregts *et al*., 2000; Simonsen *et al*., 2001) to screening of (chemo)genetic libraries (Zewail *et al*., 2003). In fact, a major motivation for the establishment of conditions that allow parallel determination of phospholipids and sphingolipids in yeast is the aim to screen existing libraries, such as the EUROSCARF collection of (non-essential) deletion strains. It can be anticipated that careful examination of phospholipid and sphingolipid profiles of yeast strains involved in sphingolipid biosynthesis (and metabolism) will yield new insights into the cross-talk between these two classes of lipids. Indeed, there is a growing body of evidence which suggests that sphingolipid and phosphatidylinositol lipid metabolism are functionally interconnected (Dickson *et al*., 1990; Nagiec *et al*., 1993, 2003; Lester *et al*., 1993; Kearns *et al*., 1998; Huijbregts *et al*., 2000). *slc1-1*, in the absence of LCB, produces inositol-containing glycerophospholipids which can rescue the growth phenotype otherwise observed (Lester *et al*., 1993).

An important 'by-product' of the results presented here is a better understanding of possibly "non-essential lipids" that are able to support growth despite alterations in their levels. The two deletion strains Δ*scs7* and Δ*slc1*, for example, are able to grow (in YPD) yet display alterations in phospholipid and sphingolipid levels which range up to two-fold. Indeed, deletion of *SCS7* almost completely abolishes the 'C series' of inositol ceramides (Figure 6D). Overlay of genetic libraries with pharmacological treatments offers enormous potential for the identification of pathways that are required for a functional condition, such as sensitivity to wortmannin (Zewail *et al*., 2003), calcium (Ohya *et al*., 1986), heat shock (Jenkins *et al*.,

1997; Ferguson-Yankey *et al*., 2002) or control of cell growth (Kunz *et al*., 1993). It will be interesting, for example, to use this method to analyse the precise lipid inventory of the strains used in this study when grown under calcium stress and in the absence of LCB precursors, rather than growth under fully permissive conditions (Dunn *et al*., 1998). This could lead to the discovery of lipid entities which are closely correlated with the calcium phenotype and which would yield important molecular information on mechanisms of action.

Phospholipids and sphingolipids are two major classes of membrane lipids found in yeast. In the future, it will be important to include sterol and non-polar lipids, such as diacylglycerols and triacylglycerols, in screens such as the one presented here. However, this will likely require different modes of ionization (such as atmospheric chemical pressure ionization) as well as additional extraction procedures, as these lipids are much more non-polar than phospholipids and sphingolipids.

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