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ORIGINAL ARTICLE

Identification and Characterization of Phospholipids with Very Long Chain Fatty Acids in Brewer's Yeast

Tomáš Řezanka[1] · Irena Kolouchová[2] · Lucia Gharwalová[2] · Andrea Palyzová[1] · Karel Sigler[1]

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Abstract Yeast lipids and fatty acids (FA) were analyzed in *Saccharomyces pastorianus* from seven breweries and in the dietary yeast supplement Pangamin. GC–MS identified more than 30 FA, half of which were very-long chain fatty acids (VLCFA) with hydrocarbon chain lengths of ≥22 C atoms. Positional isomers ω-9 and ω-7 were identified in FA with C18–C28 even-numbered alkyl chains. The most abundant ω-7 isomer was *cis*-vaccenic acid. The structure of monounsaturated FA was proved by dimethyl disulfide adducts (position of double bonds and *cis* geometric configuration) and by GC–MS of pyridyl carbinol esters. Ultra-high performance liquid chromatography-tandem mass spectrometry with negative electrospray ionization identified the phospholipids phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine, with more than 150 molecular species. Wild-type unmutated brewer's yeast strains conventionally used for the manufacture of food supplements were found to contain VLCFA.

Keywords Yeast · Very long chain fatty acids · Negative electrospray ionization · Phospholipids

Abbreviations

CID	Collision induced dissociation
DMDS	Dimethyldisulfide
DMPtdEtn	Dimethyl-phosphatidylethanolamine
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
FA	Fatty acids
FAME	Fatty acid methyl esters
FID	Flame ionization detector
FT	Fourier transform
GC–MS	Gas chromatography–mass spectrometry
HESI	Heated electrospray interface
HPLC	High-performance liquid chromatography
IT-TIC	Ion trap-total ion current
MMPtdEtn	Monomethyl-phosphatidylethanolamine
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdIns	Phosphatidylinositol
PtdSer	Phosphatidylserine
SIM	Selected-ion monitoring
TAG	Triacylglycerol
HPLC	High performance liquid chromatography

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✉ Tomáš Řezanka
rezanka@biomed.cas.cz

Irena Kolouchová
Irena.Kolouchova@vscht.cz

Lucia Gharwalová
lucia.gharwalova@gmail.com

Andrea Palyzová
palyzova@biomed.cas.cz

Karel Sigler
sigler@biomed.cas.cz

[1] Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic

[2] Department of Biotechnology, University of Chemistry and Technology Prague, Technická 5, 166 28 Prague, Czech Republic

HPLC–MS^2/ESI$^-$	High performance liquid chromatography–tandem mass spectrometry negative electrospray ionization
VLCFA	Very-long chain fatty acids

Introduction

Yeasts, especially those that are a byproduct after beer production, are often used as a dietary supplement, whether by humans or by livestock under different names such as Natural Brewers Yeast, Bierhefe Tabletten, Brewers Yeast Tablets, Brewer's Yeast, Debittered Brewer's Yeast, Pangamin, etc. Although their yeast content, including the content of fatty acids (FA) has been described in many reviews [1–6], no one has so far paid attention to the content of very long chain fatty acids (VLCFA). More than 40 years ago, Welch and Burlingame [7] described the presence of these acids up to tetratriacontanoic acid in the yeast species *Saccharomyces cerevisiae*, but monounsaturated FA only up to 28:1 (octacosenoic acid) were identified. Other papers dealing with VLCFA in yeast and specifically *S. cerevisiae,* an important microorganism on which the production of beer and wine is based [8, 9], were published only sporadically. Studies on the effect of yeast as a food supplement on the metabolism of both experimental animals and humans [10–12] were published only exceptionally.

Examples of lipids present in yeast are 1-melissoyl-2-oleolyl-*sn*-glycero-3-phosphatidylcholine (30:0/18:1-PtdCho) and 1-melissoyl-2-oleolyl-*sn*-glycero-3-phosphatidylethanolamine (30:0/18:1-PtdEtn) from fission yeast mutant *Schizosaccharomyces pombe* [13]. *Saccharomyces cerevisiae* contains about 1% of phosphatidylinositol (PtdIns) species such as 26:0 substituted PtdIns [14]. This 26:0 acid was bound exclusively in the *sn*-1 position of the glycerol backbone. In wild-type yeast, the authors identified only a few molecular species (e.g. 23:0/18:1, 26:0/16:1, 26:0/14:0, etc.) of PtdIns but not of other phospholipids such as PtdCho, PtdEtn, and/or phoshatidylserine (PtdSer). Also, in our previous article [15], we identified triacylglycerols (TAG) with VLCFA, for example 26:0/18:1/18:1, 26:0/18:1/16:0, 26:0/18:2/16:0, or 24:0/18:1/18:1. The detailed knowledge of the VLCFA content and in particular their content in the individual lipid classes, i.e. in previously published TAG and in phospholipids (this article), allows the use of yeast after the production of beer as an alternative source of VLCFA and the possibility to produce both very long chain alcohols and wax esters. This pathway is far more economically feasible than culturing yeast directly to obtain both long chain alcohols [16] and wax esters as substitutes for jojoba oil [17]. It is necessary to realize that, from the economic viewpoint, yeast is actually waste after the production of beer and the cost of its cultivation is already part of the cost of beer production. Essentially, it is thus free of charge and some breweries are even paying for its ecological disposal.

The biosynthesis of VLCFA is catalyzed by the enzyme system of fatty acid elongase that is similar to another complex, fatty acid synthase, except that it does not use acetyl-CoA as a starting substrate but moderate to long acyl-CoA which are extended up to VLCFA [18, 19]. The mechanism of its action was mostly determined using various mutant strains- in essence, it always includes blocking certain enzymes of fatty acid biosynthesis. For instance *HpELO1*, a fatty acid elongase gene encoding a 319-amino-acid protein, was identified in the methylotrophic yeast *Hansenula polymorpha* and it is an ortholog of the *S. cerevisiae ELO3* gene that is involved in the elongation of VLCFA [20]. Also other yeast mutants lacking endogenous *de novo* fatty acid synthesis were used for the purpose [21]. The study showed that the specific activity of fatty acid elongation is about 10 to 20-fold lower than that of *de novo* fatty acid synthesis, acyl-CoA with 12–14 carbon atoms being used as starter units for elongation. The practical application of the knowledge about the biosynthesis of VLCFA enabled, e.g., Wenning *et al*. [17] to perform the biosynthesis of very long-chain fatty alcohol and a wax ester in *S. cerevisiae* as a substitute of jojoba oil.

In contrast to e.g. mammals [22], with the few exceptions described above [13, 14] lipids containing VLCFA have not been localized in yeast even in studies dealing with lipidomic yeast analysis [23, 24]. For this reason, as a sequel to our work on TAG [15], we analyzed the comprehensive lipidomic profiles, focusing on polar lipids in industrially important strains of *S. cerevisiae* from seven breweries in the Czech Republic used directly for the production of beer.

Materials and Methods

Chemicals and Standards

Acetonitrile, 2-propanol, hexane, dichloromethane, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine and ergosterol were purchased from Sigma-Aldrich (Prague, Czech Republic). Ergosteryl oleate, triolein, diolein, oleic acid, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine, 1,2-dioleoyl-*sn*-phosphatidylethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (sodium salt), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid, Na-salt, and 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl-rac-glycerol (Na-salt), were from Larodan (Malmö, Sweden). 1,2-Dioleoyl-*sn*-glycero-3-phosphatidyl-(1′-myo-inositol) (ammonium salt) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl-(1′-myo-inositol-3′-phosphate) (ammonium salt), and 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl-(1′-myo-inositol-3′,5′-disphosphate)

(ammonium salt), were purchased from Avanti (Avanti Polar Lipids, Inc., Alabama, USA).

Collection of Yeast

Bottom brewer's yeast *S. pastorianus* was obtained from selected breweries (see Table 1S). Pangamin was purchased at a local pharmacy.

Isolation of Lipids

The extraction and isolation of total lipids from the yeast suspension was performed according to a previously described methodology [25]. Briefly, the yeast suspension (10 mL, approximately 15% of dry mass) was ground with glass beads under liquid nitrogen. After thawing, the crushed yeast was extracted in a blender (5 min) according to Bligh and Dyer [26] (10 mL chloroform and 20 mL of 2-propanol) of except that 2-propanol was substituted for methanol, since 2-propanol does not serve as a substrate for phospholipases.

Analysis of Fatty Acid Methyl Esters and Dimethyldisulfides

FAME were prepared after saponification and further esterification [15] using BF_3/MeOH. The FAME (~1 mg) were dissolved in dimethyldisulfide (DMDS) (0.2 mL) and a solution of iodine in diethyl ether (3 mg in 0.05 mL) was added. The mixture was stirred for 1 day, then hexane (5 mL) was added and the mixture was washed with dilute sodium thiosulfate solution, dried over anhydrous sodium sulfate and evaporated. The products were dissolved in hexane and analyzed by GC–MS [27, 28].

GC–MS of FAME was done on a Varian Saturn Ion Trap 2000 GC/MS/MS system with CP (Combi PAL autosampler)-3800 gas chromatograph system with the split/splitless injector (250 °C) and a SP™-2380 Capillary GC Column L × ID 60 m × 0.25 mm, *df* 0.20 µm capillary column. Helium was used as the carrier gas at 1.0 mL/min. The split/splitless injection port was maintained at 255 °C. The split ratio was 1:90, and the injection volume was 1 µL. For FAME GC–MS analysis with the SP-2380 column, the temperature program was as follows: 150 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 250 °C, which was maintained for 1 min. A higher oven temperature was used to separate the DMDS—the starting temperature was 180 °C for 1 min, subsequently increasing at 20 °C/min to 220 °C and at 2 °C/min to 260 °C, which was maintained for 1 min. FAME were identified according to their mass spectra [29, 30] and using a mixture of chemical standards obtained from Sigma-Aldrich. All experiments concerning the analysis of FAME and their derivatives were carried out by electron impact mass spectrometry. Mass spectra were recorded on a GC–MS system consisting of a Varian 450-GC, a Varian 240-MS ion trap detector with electron impact ionization (70 eV), and a CombiPal autosampler (CTC, USA). All spectra were scanned within the range *m/z* 50–600. The structures of FAME were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAME (Supelco, Czech Republic). The MS detector operated at 194 °C and solvent delay was 10 min. Calculations of the amount of FA were carried out by software (MS Workstation, v. 6.9, Varian) supplied with the device on the basis of total ion current (as relative area).

Polar Normal Phase Chromatography LC/MS–ESI

The HPLC equipment consisted of a 1090 Win system, a PV5 ternary pump and an automatic injector (HP 1090 series, Hewlett Packard, USA), and an Ascentis® Express OH5, HPLC column (2.7 µm particle size, L × ID 15 cm × 2.1 mm) (Supelco, Prague, Czech Republic) with an injection volume of 10 µL. HPLC was performed at a flow rate of 0.33 mL/min with a linear gradient from the mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (50:30:20, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (10:70:20, v/v/v) for 60 min. Column temperature was 35 °C and a re-equilibration period between runs was 30 min. The whole HPLC flow was introduced into the ESI source without any splitting.

LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used. ESI–MS analysis was performed in the FT negative ion mode. MS spectra were acquired with target mass resolution of $R = 100{,}000$ at *m/z* 400. The ion spray voltage was set at −2500 V and the scan range of the instruments was set at *m/z* 200–2000. Nitrogen was used as a nebulizer gas—set at 18 arbitrary units (sheath gas) and 7 arbitrary units (aux gas). Helium was used as a collision gas for collision induced dissociation (CID) experiments. The CID normalization energy of 35% was used for the parent ions fragmentation. The MS/MS product ions were detected by the high resolution Fourier transform (FT) mode. The HESI temperature was set at 250 °C.

Results

Fatty Acids

Table 1 shows the fatty acid content of yeast obtained from seven breweries and a commercially produced dietary supplement Pangamin. GC–MS analysis of total FAME

Table 1 Fatty acid composition (% of total FA), determined by GC–MS of FAME from seven breweries and dietary supplement (Pangamin)

Fatty acid	Brewery A	SD[a]	Brewery B	SD	Brewery C	SD	Brewery D	SD	Brewery E	SD	Brewery F	SD	Brewery G	SD	Pangamin	SD	SD[b]
8:0	n.d.	**n.d.**	n.d.	**n.d.**	0.112	**±0.009**	n.d.	**n.d.**	0.607	**±0.008**	n.d.	**n.d.**	n.d.	**n.d.**	n.d.	**n.d.**	*±0.199*
10:0	n.d.	**n.d.**	2.336	**±0.021**	1.341	**±0.013**	n.d.	**n.d.**	3.917	**±0.012**	n.d.	**n.d.**	n.d.	**n.d.**	2.312	**±0.012**	*±1.400*
12:0	n.d.	**n.d.**	2.248	**±0.014**	3.110	**±0.022**	3.418	**±0.011**	1.341	**±0.011**	4.044	**±0.014**	4.337	**±0.016**	2.243	**±0.014**	*±1.352*
14:0	n.d.	**n.d.**	0.982	**±0.009**	0.571	**±0.009**	1.442	**±0.009**	1.954	**±0.015**	1.768	**±0.011**	1.707	**±0.008**	0.870	**±0.009**	*±0.631*
16:0	9.441	**±0.012**	11.521	**±0.027**	20.098	**±0.024**	23.507	**±0.026**	22.614	**±0.026**	17.512	**±0.024**	17.848	**±0.023**	11.506	**±0.024**	*±5.016*
9–16:1 or 16:1ω-7	43.625	**±0.031**	41.874	**±0.029**	43.673	**±0.028**	36.762	**±0.027**	36.663	**±0.028**	39.469	**±0.028**	44.054	**±0.026**	41.937	**±0.029**	*±2.831*
18:0	4.076	**±0.014**	5.336	**±0.014**	6.812	**±0.014**	13.879	**±0.021**	9.678	**±0.019**	17.472	**±0.02**	13.915	**±0.028**	5.341	**±0.022**	*±4.657*
9–18:1 or 18:1ω-9	31.836	**±0.042**	26.809	**±0.019**	21.364	**±0.021**	15.831	**±0.025**	15.473	**±0.017**	14.486	**±0.026**	13.211	**±0.023**	26.836	**±0.019**	*±6.568*
9,12–18:2 or 18:2ω-6	3.455	**±0.010**	0.962	**±0.008**	0.332	**±0.009**	3.325	**±0.014**	2.774	**±0.009**	1.708	**±0.009**	1.167	**±0.011**	0.982	**±0.013**	*±1.115*
20:0	0.217	**±0.008**	0.183	**±0.009**	0.094	**±0.006**	n.d.	**n.d.**	0.342	**±0.005**	1.125	**±0.008**	1.217	**±0.014**	0.227	**±0.009**	*±0.441*
11–20:1 or 20:1ω-9	1.238	**±0.009**	1.491	**±0.022**	n.d.	**±n.d.**	n.d.	**n.d.**	0.448	**±0.006**	n.d.	**±n.d.**	n.d.	**n.d.**	1.463	**±0.007**	*±0.652*
22:0	0.670	**±0.013**	0.873	**±0.014**	n.d.	**±n.d.**	n.d.	**n.d.**	0.791	**±0.005**	n.d.	**±n.d.**	n.d.	**n.d.**	0.907	**±0.008**	*±0.410*
13–22:1 or 22:1ω-9	0.518	**±0.011**	0.811	**±0.013**	n.d.	**±n.d.**	n.d.	**n.d.**	0.567	**±0.008**	n.d.	**±n.d.**	n.d.	**n.d.**	0.844	**±0.005**	*±0.357*
24:0	0.469	**±0.008**	0.578	**±0.011**	n.d.	**±n.d.**	n.d.	**n.d.**	0.686	**±0.007**	n.d.	**±n.d.**	n.d.	**n.d.**	0.572	**±0.006**	*±0.293*
15–24:1 or 24:1ω-9	0.772	**±0.005**	0.990	**±0.017**	n.d.	**±n.d.**	n.d.	**n.d.**	0.390	**±0.005**	n.d.	**±n.d.**	n.d.	**n.d.**	1.023	**±0.010**	*±0.435*
26:0	0.743	**±0.009**	0.904	**±0.010**	0.086	**±0.014**	n.d.	**n.d.**	n.d.	**n.d.**	n.d.	**±n.d.**	n.d.	**n.d.**	0.871	**±0.008**	*±0.401*
17–26:1 or 26:1ω-9	0.913	**±0.015**	0.827	**±0.009**	0.707	**±0.009**	0.438	**±0.008**	0.518	**±0.006**	0.882	**±0.011**	0.936	**±0.006**	0.844	**±0.009**	*±0.175*
28:0	0.929	**±0.014**	0.421	**±0.005**	0.682	**±0.010**	0.459	**±0.007**	0.632	**±0.008**	0.694	**±0.009**	0.642	**±0.008**	0.465	**±0.006**	*±0.156*
19–28:1 or 28:1ω-9	0.386	**±0.009**	0.342	**±0.008**	0.412	**±0.008**	0.375	**±0.009**	0.269	**±0.009**	0.383	**±0.005**	0.361	**±0.006**	0.392	**±0.004**	*±0.041*
30:0	0.327	**±0.006**	0.175	**±0.006**	0.317	**±0.006**	0.381	**±0.006**	0.083	**±0.004**	0.171	**±0.004**	0.229	**±0.005**	0.183	**±0.005**	*±0.094*
21–30:1 or 30:1ω-9	0.274	**±0.008**	0.256	**±0.003**	0.190	**±0.007**	0.191	**±0.004**	0.182	**±0.003**	0.286	**±0.005**	0.324	**±0.008**	0.182	**±0.006**	*±0.053*
32:0	0.111	**±0.005**	0.081	**±0.004**	0.099	**±0.004**	0.092	**±0.005**	0.071	**±0.005**	n.d.	**n.d.**	0.052	**±0.002**	n.d.	**n.d.**	*±0.040*

[a] Arithmetic means from three analysis ± standard deviations (bold)

[b] Arithmetic means from 7 breweries and Pangamin ± standard deviations (italic)

obtained from yeast from breweries A is shown in Fig. 1S, which shows that a total of 24 FAME up to 32:0 were separated and identified. It is evident that the differences in the contents of FA between different breweries are not large, which is understandable since the yeast is in all cases essentially a wild-type yeast, i.e. yeast cells that have not genetically mutated. Table 1 and Fig. 1S show that in the case of monoenoic FA, more than one positional isomer exists for FA with the same carbon number.

FAME analysis by two different methods of quantification, i.e. an ion trap-total ion current (IT-TIC) and a flame ionization detector (FID), was also performed in a commercially obtained standard (NHI-D FAME mix, Sigma, Czech Republic). It has been found that for quantification of the majority of FAME, for example for 18:1, MS quantification was closer to the weight% of this methyl ester in the standard than FID quantification. In addition, from Table 1 and 3S it is clear that the differences between breweries (±SD) are often up to two orders of magnitude higher than the differences between the detectors. When the ±SD value between individual analyses for the same detector was around 0.1, then ±SD for individual breweries analyses is up to two orders of magnitude higher. It follows from the above that the variability of the samples from the biological point of view (i.e. between the individual FAME in the sample), for is the same yeast (genus and species), is much higher than the differences between the individual detection techniques. In addition, from both our (Table 3S) and published data [31] it is clear that IT-TIC detection corresponds more closely to the actual FAME representation in the analyzed sample.

One should note that all breweries do not contain all the FA, e.g. the content of FA 26:0 in breweries D–G is below 0.1% of the total fatty acids. Similarly the representation of the individual molecular species in PtdEtn, PtdCho, PtdIns, in Tables 3, 4aS and 4bS differs up to several orders. As an example, FA 26:0 in breweries A, B, C and Pangamin is present in 1% of total FA, in D–G breweries it is below 0.1% of the total FA. The molecular species analysis shows that the proportion of molecular species of 26:0/26:0-PtdCho in breweries A, B, C and Pangamin ranges from ~7 to ~10%, whereas in breweries D–G the content of this molecular species is one to two orders lower (0.021–0.140%).

Octadecenoic acid is present as several positional isomers such as Δ^9 and Δ^{11}, i.e. oleic and *cis*-vaccenic acids. To prove the presence of both acids, including the configuration of the double bond, four commercially obtained standards, i.e. oleic, elaidic, *cis*-vaccenic and vaccenic acids were converted to the corresponding DMDS adducts. Comparison of the mass spectrum of the standard and FAME derived from brewer's yeast (see below) showed the presence of oleic and *cis*-vaccenic acids. DMDS adduct formation is stereospecific, which means that the addition to the *cis* double bond gives rise to the *threo*-, and addition to the *trans* double bond to the *erythro*-isomer [32]. Although both derivatives have identical mass spectra, they differ in the retention time in the GC–MS, the *threo*-isomer having a shorter retention time. Figures 2S and 3S show DMDS adducts of two FAME, i.e. natural oleic and *cis*-vaccenic acids. In each mass spectrum are diagnostic ions $[M]^+$ at *m/z* 390.2 and ions generated by cleavage of single bonds, see the inset in each mass spectrum. On the basis of these two mass spectra we unequivocally showed that the compounds are 9–18:1 and 11–18:1. The geometric configuration was determined to be *cis* based on the conformity of the retention times of the adducts with the adducts prepared from commercially available standards.

To prove further the presence of positional isomers of monounsaturated fatty acids other than ω-9, the total FAME were converted to DMDS adducts and analyzed by GC–MS. Figure 1 shows the selected-ion monitoring (SIM) chromatogram for ions at *m/z* 145 (top trace) for ω-7 FA, and at *m/z* 173 (lower recording) for ω-9 FA. Figure 4S gives an original recording (blank) as well as the ion at *m/z* 117, i.e. ω-5 FA. This could reflect, e.g., the presence or absence of 16:1ω-5 acid (11-hexadecenoic acid), which could arise by desaturation of palmitic acid by the Δ^{11} desaturase, i.e. analogously to the formation of *cis*-vaccenic acid from stearic acid. Unfortunately, we could not prove any ω-5 FA. Figure 4S further shows that the abundance of ω-7 monoenoic FA *versus* ω-9 FA is much lower, much like the situation in the pair oleic *versus cis*-vaccenic acid.

Further confirmation of the structure, i.e. the position of a double bond in VLCFA, was performed using 3-pyridyl carbinyl (formerly picolinyl) esters. Octacosenoic acid (28:1) was chosen as an example. In the interval of low *m/z* values, as with any other 3-pyridyl carbinyl esters, there are abundant ions at *m/z* 92, 108, 151, 164, etc. which are not diagnostically significant. In both 3-pyridyl carbinyl esters, the $[M]^+$ ion had the value of 513.5 Da. As is evident from the GC–MS of DMDS adducts, these are two positional isomers of 28:1 acids. We assumed and then confirmed (see below) that they are 28:1ω-7 (Fig. 5S) and 28:1ω-9 (Fig. 6S). The mass spectrum of 19–28:1 (28:1ω-9) contains diagnostic ions, i.e. the ions that determine the position of a double bond, which have values of 374.3 and 400.3 Da. The difference between them is the gap 26 Da which corresponds to –CH=CH– group. More abundant ions of the gap 40 Da are those at *m/z* 360.3–400.3. Similarly, 21–28:1 (28:1ω-7) showed a 26 Da gap between ions at *m/z* 402.3 and *m/z* 428.3, and 40 Da gap between *m/z* 388.3 and 428.3, which is more distinct (the ions have a higher abundance). The above analysis clearly shows that the wild-type strains contain both homologous series of monounsaturated FA, i.e. ω-7 and ω-9.

We thus identified even-numbered monounsaturated ω-7 FA with 18–28 carbon atoms in the chain. As far as we

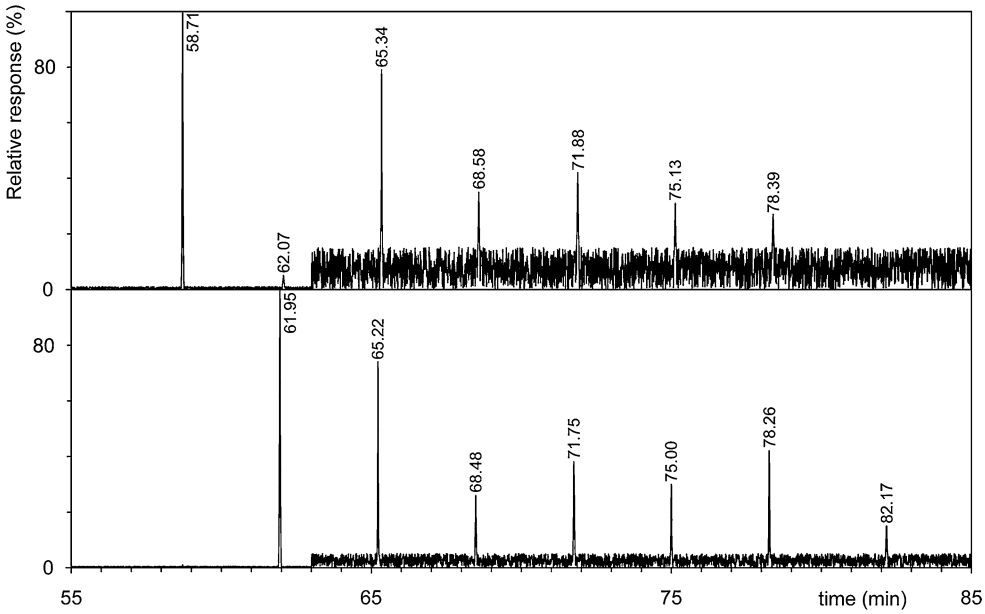

Fig. 1 GC–MS analysis of total FAME as DMDS adducts obtained from yeast from brewery A shows a selected-ion monitoring chromatogram for ions at *m/z* 145 (top trace) for ω-7 FA, and at *m/z* 173 (lower recording) for ω-9 FA

know, this is the first case when positional isomers of more than one monounsaturated FA have been confirmed in yeast.

Polar Lipids

Separation of commercially obtained lipid standards and lipids from seven breweries and Pangamin (see "Materials and Methods") was performed by gradient elution on an HPLC column with OH groups. Base-line separation of both standards and real samples by HPLC was achieved within 1 h, see Fig. 2. Lipids were detected as $[M+CH_3COO]^-$ (only PtdCho) and $[M–H]^-$ ions. A partial intra-class separation occurred in some cases- it was unfortunately not sufficient and we did not use it. Fragmentation of phospholipids includes mostly deprotonation of molecular ion $[M–H]^-$ and the formation, in tandem

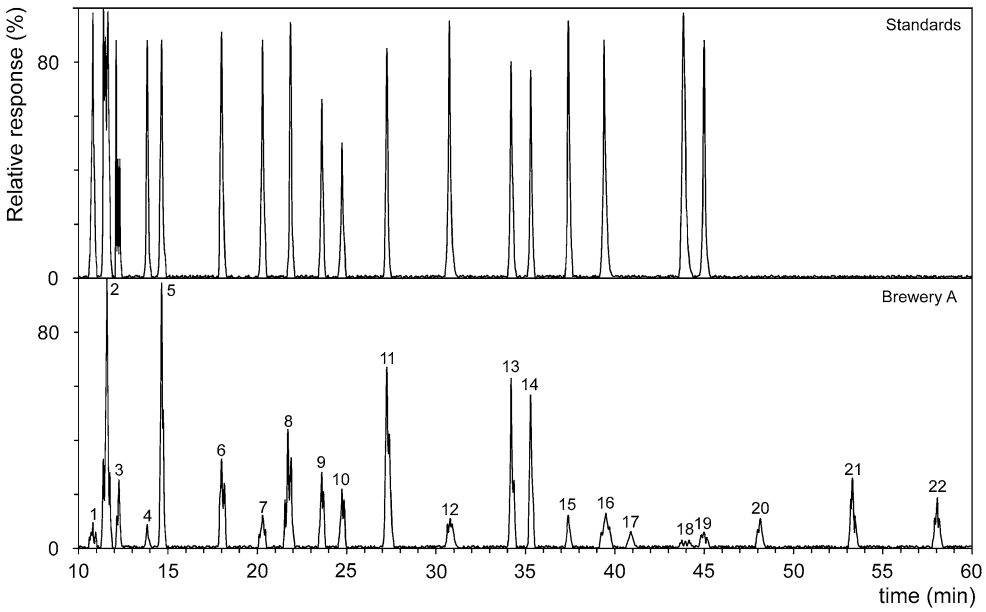

Fig. 2 Separation of both standards and real sample from brewery A by HPLC column with negative electrospray ionization, *1*—steryl ester, *2*—triacylglycerol, *3*—diacylglycerol, *4*—unesterified fatty acid, *5*—sterol, *6*—phosphatidylglycerol, *7*—cardiolipin; *8*—phosphatidylcholine, *9*—dimethyl-phosphatidylethanolamine, *10*—monomethyl-phosphatidylethanolamine, *11*—phosphatidylethanolamine *12*—lyso-phosphatidylcholine, 13—phosphatidylinositol, *14*—phosphatidic acid, *15*—phosphatidylserine, *16*—phosphatidylinositol phosphate, *17*—unknown, *18*—lyso-phosphatidic acid, *19*—unknown; 20—unknown, *21*—unknown; *22*—unknown

MS (MS/MS), of ion types arising by, e.g., neutral loss of free fatty acid [(M–H–RCOOH)$^-$], neutral loss of fatty acyl group as a ketene [(M–H–R′CH=C=O)$^-$], and fatty carboxylate anion [(RCOO)$^-$]. Regioisomers (molecular species differing only in the location of acyls at *sn*-1 and *sn*-2 positions) can be identified on the basis of relative intensities of [M–H–R'CHCO]$^-$ ions.

Phosphatidylcholine

PtdCho contains a quaternary amino group and therefore for its negative ionization ESI the eluate must be spiked by, e.g., acetate buffer. PtdCho then forms ions of the type [M+CH_3COO]$^-$. In our case, one of PtdCho was detected as acetate anion adduct under MS/MS at *m/z* 1180.9829 and its spectrum showed major peaks at *m/z* 1106.9458 (loss of methyl and acetate from precursor ion), and at *m/z* 499.4364 [($C_{29}H_{57}COO$)$^-$]. Other diagnostic ions permitting the determination of the structure of the molecular species is ion at *m/z* 674.5131 [M–15–$C_{28}H_{55}CH$=C=O]$^-$ formed by loss of ketene, or ion arising by neutral loss of free FA at *m/z* 656.5025 [M–15–$C_{28}H_{55}CH_2COOH$]$^-$. Other ions present in the spectrum, i.e. ions at 1035.8724 Da (loss of choline and acetate from precursor ion), at 224.0693 Da (glycerophosphocholine with loss of CH_3 and H_2O), and ion at 168.0431 Da (phosphocholine with loss of CH_3), and ion at 152.9958 Da (glycerol-3-phosphate ion with loss of H_2O) fully support the proposed structure of 30:1/30:1-PtdCho. Table 2S gives the relative percentage of PtdCho containing VLCFA in the molecule.

Phosphatidylinositol

In the negative spectrum of molecular species of the PtdIns, where the ion [M–H]$^-$ ion has a value of 1169.8942 Da under MS/MS, are also present additional ions, i.e. three pairs formed by loss of acyl chains as ketene (R'CH=C=O) from [M–H]$^-$ (ions at 765.4923 and 737.4611 Da), by neutral loss of RCOOH [(M–H–RCOOH)$^-$] (ions at 747.4817 and 719.4505 Da), and fatty carboxylate anions [(RCOO)$^-$] (ions at 449.4364 and 421.4051 Da). Other present ions are derived from inositol polar head group at *m/z* 1007.8413 [loss of inositol from (M–H)$^-$], at *m/z* 315.0487 and 297.0381, respectively (glycerophosphoinositol losing one or two molecules of water). The above values are fully consistent with the proposed structure *sn*-30:1/28:1-PtdIns. All molecular species of other PtdIns were identified in a similar way. Other sufficiently abundant ions, including of their structures, are listed in Table 3S.

Phosphatidylethanolamine

For PtdEtn we chose such [M–H]$^-$, which contained three different molecular species, as can be seen from the following ions generated by MS/MS fragmentation. Based on ions of the type [RCOO]$^-$, i.e. six ions, we identified the following acyl chains: 22:0, 22:1, 24:0. 24:1, 26:0 and 26:1, see Fig. 3. Evidence that this is PtdEtn was provided by the presence of ions at *m/z* 152.9958 (glycerol-3-phosphate ion with loss of H_2O) and at *m/z* 140.0118 (ethanolamine phosphate ion). The complete structure of respective molecular species has been demonstrated by a series of 12 ions, i.e. three ions arising from loss of the *sn*-2 acyl chain with ketene (R–CH=C=O) from [M–H]$^-$ at

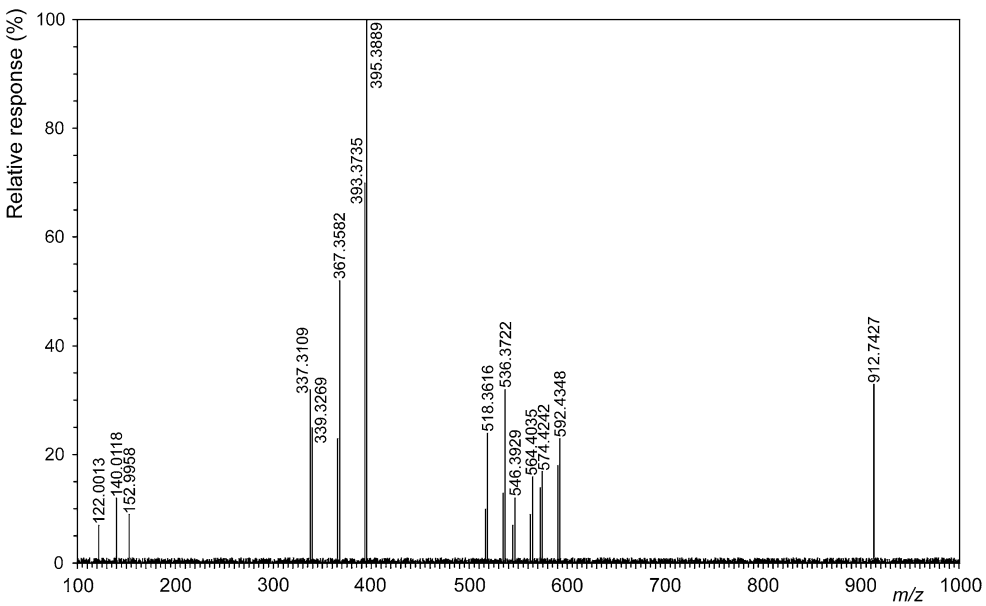

Fig. 3 High resolution tandem mass spectra of [M–H]$^-$ ions at *m/z* 912.7427 (molecular species of phosphatidylethanolamine 26:0/22:1, 24:0/24:1 and 26:1/22:0) from lipid extract of yeast from brewery A

m/z 592.4348, 564.4035, and 536.3722 and, in addition, three ions of the type $[M–H–R_2COOH]^-$ at *m/z* 574.4242, 546.3929, and 518.3616. The structure was also confirmed by the ions arising from loss of *sn*-1-acyl chain and with ketene (R–CH=C=O) from $[M–H]^-$, or three ions of the type $[M–H–R_1COOH]^-$ whose values are listed in Table 2. Based on the intensities of the above diagnostic ions we found that the acyl chain at position *sn*-1 is always longer, or at least as long, as that at *sn*-2. The results of analysis thus show the presence of three molecular species, namely two major 26:0/22:1-PtdEtn and 26:1/22:0-PtdEtn, and minor 24:0/24:1-PtdEtn. Representation of abundant molecular species containing VLCFA is shown in Table 3.

Discussion

Fatty Acids

The literature data on the presence or absence of VLCFA in *S. cerevisiae* lipids are somewhat controversial. According to several authors, yeast did not reveal any FA with more than 18 carbon atoms [24, 33, 34]. In strain BY4741 saturated even-chain C20–C26 FA were found only in sphingolipids and TAG [35].

On the other hand, something else was observed in older studies, which were fully focused on whether VLCFA are present in yeast or not. In the first of these, the study by Welch and Burlingame [7] performed more than 40 years ago, the authors identified odd- and even-numbered monoenoic acids of up to 28:1 and saturated FA up to 34:0. Also the presence of *cis*-vaccenic acid, or the presence of Δ^{11} desaturase, has been demonstrated several times. Thus Sec *et al*. [36] have found in a wild strain (BY4742) *cis*-vaccenic acid in an amount more than one order of magnitude lower than that of oleic acid. Southwell-Keely and Lynen [37] described the presence of *cis*-vaccenic acid in a wild strain S288Cα when the yeast grew on decanoic and tetradecanoic acids- hence, Δ^{11}-desaturase had to be present. Augustyn and Kock [38] in their work on FA in *S. cerevisiae* found the presence of *cis*-vaccenic acid in 13 strains in the range of several percent based on the content of oleic acid.

Polar Lipids

Identification of neutral lipids in brewer's yeast has already been described [15], so in this article we shall focus on polar lipids, mainly phospho- and glycolipids, which constitute an essential part of lipids in non-oleogenic yeasts [39] that include also the genus *Saccharomyces*. Similarly as in a previous publication [25], total lipids were analyzed by LC/MS on a column in negative electrospray mode.

Hein and Hayen [40] were among the few authors who mentioned molecular species of MMPtdEtn and

Table 2 Product ions for molecular species of 26:0/22:1-PtdEtn (normal font), **24:0/24:1-**PtdEtn (bold), and *26:1/22:0*-PtdEtn (italics)

HR-MS	Abundance (%)	26:0/22:1-PtdEtn, **24:0/24:1-PtdEtn**, and *26:1/22:0-PtdEtn*
122.0013	7	Ethanolamine phosphate ion with loss of H_2O
140.0118	12	Ethanolamine phosphate ion
152.9958	9	Glycerol-3-phosphate ion with loss of H_2O
337.3109	32	sn-2 $RCOO^-$ ion
339.3269	*99*	*sn-1 $RCOO^-$ ion*
365.3425	**23**	**sn-2 $RCOO^-$ ion**
367.3582	**52**	**sn-1 $RCOO^-$ ion**
393.3735	*45*	*sn-2 $RCOO^-$ ion*
395.3889	72	sn-1 $RCOO^-$ ion
516.3460	10	Neutral loss of sn-1 RCOOH group from $[M–H]^-$
518.3616	*24*	*Neutral loss of sn-2 RCOOH group from $[M–H]^-$*
534.3565	13	Loss of sn1 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
536.3722	*32*	*Loss of sn-2 acyl chain as ketene (RCH=C=O) from $[M-H]^-$*
544.3773	**7**	**Neutral loss of sn-1 RCOOH group from $[M–H]^-$**
546.3929	**12**	**Neutral loss of sn-2 RCOOH group from $[M–H]^-$**
562.3878	**9**	**Loss of sn-1 acyl chain as ketene (RCH=C=O) from $[M–H]^-$**
564.4035	**16**	**Loss of sn-2 acyl chain as ketene (RCH=C=O) from $[M–H]^-$**
572.4085	*14*	*Neutral loss of sn-1 RCOOH group from $[M–H]^-$*
574.4242	17	Neutral loss of sn-2 RCOOH group from $[M–H]^-$
590.4191	*18*	*Loss of sn-1 acyl chain as ketene (RCH=C=O) from $[M–H]^-$*
592.4348	23	Loss of sn-2 acyl chain as ketene (RCH=C=O) from $[M–H]^-$
912.7427	33	Precursor ion $[M–H]^-$

Table 3 Molecular species of PtdEtn containing VLCFA from brewery A

Mol spec[a]	Abundance	Mol spec	Abundance	Mol spec	Abundance	Mol spec	Abundance	Mol spec	Abundance	Sum of mol spec	$[M–H]^-$ (Da)
22:1/22:1[b]	1.2									1.2	854.6644
22:1/22:0	3.4									3.4	856.6801
22:0/22:0	9.5									9.5	858.6957
22:1/24:1	2.5									2.5	882.6957
22:1/24:0	6.2	22:0/24:1	7.2							13.4	884.7114
22:0/24:0	17.2									17.2	886.7271
22:1/26:1	4.4	24:1/24:1	5.4							9.8	910.7270
22:1/26:0	9.3	22:0/26:1	12.3	24:1/24:0	6.2					27.8	912.7427
22:0/26:0	30.9	24:0/24:0	13.9							44.8	914.7584
22:1/28:1	2.1	24:1/26:1	9.2							11.3	938.7583
22:1/28:0	3.0	22:0/28:1	5.8	24:1/26:0	23.3	24:0/26:1	22.1			54.2	940.7740
22:0/28:0	8.5	24:0/26:0	55.6							64.1	942.7897
22:1/30:1	1.1	24:1/28:1	4.4	26:1/26:1	15.8					21.3	966.7896
22:1/30:0	1.2	22:0/30:1	3.3	24:1/28:0	6.4	24:0/28:1	10.6	26:1/26:0	39.8	61.3	968.8053
22:0/30:0	3.5	24:0/28:0	15.3	26:0/26:0	81.2					100.0	970.8210
24:1/30:1	2.5	26:1/28:1	7.5							10.0	994.8209
22:1/32:0	0.7	24:1/30:0	2.6	26:1/28:0	10.9	24:0/30:1	6.0	26:0/28:1	19.1	39.3	996.8366
22:0/32:0	2.1	24:0/30:0	6.3	26:0/28:0	27.6					36.0	998.8523
26:1/30:1	4.3	28:1/28:1	3.6	24:1/32:0	1.5					9.4	1022.8522
26:1/30:0	4.5	26:0/30:1	10.9	28:1/28:0	5.2					20.6	1024.8679
24:0/32:0	3.8	26:0/30:0	11.5	28:0/28:0	7.6					22.9	1026.8836
28:1/30:1	2.0									2.0	1050.8835
28:0/30:1	2.9	26:1/32:0	2.7	28:1/30:0	2.1					7.7	1052.8992
26:0/32:0	6.9	28:0/30:0	3.1							10.0	1054.9149
30:1/30:1	1.1									1.1	1078.9148
28:1/32:0	1.2	30:1/30:0	1.2							2.4	1080.9305
30:0/30:0	1.2	28:0/32:0	1.8							3.0	1082.9461
30:1/32:0	0.7									0.7	1108.9618
30:0/32:0	0.7									0.7	1110.9774
32:0/32:0	0.4									0.4	1139.0087

[a] Molecular species

[b] For abbreviations, see Table 1

DMPtdEtn, but even in these phospholipid classes VLCFA were not identified. The study by da Silveira Dos Santos *et al*. [41], which analyzed the lipidomic profile of the wild strains BY4741, Y7092, and/or Y7220, showed the presence of VLCFA in many phospholipid classes, though in trace amounts. For instance, lysophosphatidylcholines (26:1/0:0-PtdCho or 26:0/0:0-PtdCho) were present at a concentration of 0.003%, PtdIns 48:1 or 48:0 at a concentration of 0.001% and PtdCho 48:2 in a concentration of 0.0001% based on total lipids.

A completely different situation was described in studies specifically devoted to molecular species of phospholipids containing VLCFA. Here we mention only two of these rather scanty studies. In the first of them, Yokoyama *et al*. [13] analyzed both a wild strain and a *fas2* mutant strain. While the wild strain was again found to contain MMPtdEtn, e.g. 18:1/18:1-MMPtdEtn, and further minor molecular species 16:1/18:1-MMPtdEtn, 18:1/18:1-DMPtdEtn and 16:1/18:1-DMPtdEtn, the mutant *fas2* strain (*fas2*-H518) contained also 30:0/18:1-PtdEtn and 30:1/18:1-PtdCho. In the second paper, Schneiter *et al*. [14] described the presence of PtdIns containing VLCFA with the following molecular species (23:0/18:1 26:0/16:1 26:0/14:0, etc.).

Conclusion

Based on the analyses of both FA and lipids it was found that strains of brewer's yeast contain VLCFA in amounts of a few percent of the total FA. These acids are present both in TAG [15] and in the major classes of phospholipids (PtdEtn, PtdCho and PtdIns). The main contribution of this publication can be seen in two areas. In the first one, i.e. the analytical part, it was proved that the wild strains of yeast contain fatty acids of from C8 to C32, especially the saturated FA. Furthermore, monounsaturated FA have been identified with two homologous series differing only in the position of the double bonds, i.e. minor ω-7 (excluding palmitoleic acid) and the majority of the ω-9 series, including oleic acid. Further, the individual lipid classes were separated by HPLC and tens of molecular species of PtdCho, PtdEtn and PtdIns were identified by tandem MS. We consider the results of this analysis to be important, as except for a single case [13] when only a few molecular species of PtdIns containing VLCFA from wild yeast were identified, all other molecular species of PtdCho and PtdEtn containing VLCFA [14] were obtained from yeast mutants. By demonstrating the presence of VLCFA both in total lipids and in the wild species of yeast species and in addition produced in hundreds of thousands of tons per year, we should be able to obtain either VLCFA or phospholipids containing VLCFA in many-ton quantities.

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Compliance with Ethical Standards

Conflict of interest The authors declare no competing financial interest.

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