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Research Article

Identification of regioisomers and enantiomers of triacylglycerols in different yeasts using reversed- and chiral-phase LC–MS

LC with atmospheric pressure chemical ionization (ACPI) MS with RP and chiral phase was used for separation of triacylglycerols (TAGs) from yeasts of the genera *Candida*, *Kluyveromyces*, *Rhodotorula*, *Saccharomyces*, *Torulospora*, *Trichosporon*, and *Yarrowia*. Chiral LC–APCI-MS is based on using two columns in series packed with a 3,5-dimethylphenyl carbamate modified β-cyclodextrin chiral phase. All regioisomers and enantiomers of TAGs containing one to five double bonds were separated. Molecular species of TAGs, i.e. regioisomers and enantiomers, were identified and quantified by MS/MS. Among the 94 identified TAGs, the most abundant were triolein, oleopalmitoleoolein, and dipalmitoleoolein. In strains producing palmitoleic acid in amounts >25% of total fatty acids (FAs), this acid, or unsaturated FA is bound in *sn*-1. In strains containing palmitoleic acid at 10–25% total FAs this acid is mainly bound in *sn*-3, saturated FA being bound in *sn*-1. Strains containing <10% palmitoleic acid form preferentially symmetrical TAGs.

Keywords: Atmospheric pressure chemical ionization mass spectrometry / Chiral LC / Reversed phase LC / Triacylglycerols / Yeast
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1 Introduction

The term lipokine was first used by Cao et al. [1] to classify fatty acids (FAs) that modulate lipid metabolism by what they called a chaperone effect. Palmitoleic acid (9-16:1 or 16:1ω7 or Po) has been recognized to act as a lipokine. It is derived from adipose tissue and is able to stimulate the action of insulin at the muscle level and to decrease the accumulation of lipids in the liver (hepatosteatosis) [2]. Palmitoleic acid is also a powerful activator of the adipocyte peroxisome proliferator-activated receptors, γ-receptors [3]. It has been shown that the plasma palmitoleic acid is related to abdominal adiposity in children [4]. Additionally, administering palmitoleic acid to a subject (via nutraceuticals or other means), positively impacts lipid metabolism [2].

Plant oils containing >10% of palmitoleic acid are usually obtained from relatively exotic plant sources [5]. Moreover, low yields and poor agronomic characteristics of these plants prevent the large-scale production of these oils.

By contrast, yeast is an attractive source for the bioproduction of palmitoleic and other beneficial FAs since some oily yeasts, e.g. yeasts of genera *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Trichosporon*, and *Lipomyces* [6], are able to accumulate lipids in amounts exceeding one quarter of cell dry weight. FAs are found in yeast cells either as neutral lipids, i.e. triacylglycerols (TAGs), or as polar lipids, e.g. phospholipids or glycolipids. The yeast cultivation is very well managed and the economic feasibility of the process for producing microbial lipids is determined by the cost of raw materials and the simplicity of this process. It can be improved by the selection of a suitable yeast strain and also by changing physiological and cultivation conditions suitable for preparing FAs with the required chain length (number of carbon atoms) or a required number of double bonds. Usually, four FAs, i.e. palmitic (16:0 or P), palmitoleic, stearic (18:0 or S), and oleic (9-18:1 or O) are found as major acids. The spectrum of FAs in yeast lipids includes also numerous minor constituents such as medium-length FAs (up to C_{14}) or very long chain FAs (above C_{22}) [7].

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Abbreviations: ACN, acyl carbon number; **APCI**, atmospheric pressure chemical ionization; **FAMEs**, Fatty acid methyl esters; **FAs**, fatty acids; **iPrOH**, 2-propanol; **NARP-HPLC**, nonaqueous RP-HPLC; **OOO**, triolein; ***sn*-OPoO**, oleopalmitoleoolein; **TAGs**, triacylglycerols; **NLS**, neutral-loss scan; **TIC**, total ion current

It should be noted that the relationship between the production of total lipids and the content of palmitoleic acid is not simple—the higher the production of total lipids by a yeast, the lower the proportion of palmitoleate. For instance, *Trichosporon pullulans* contains 65% lipids in dry weight but the palmitoleic acid in them has not been identified [7]. If the content of palmitoleic acid is as high as tens of percent of total FAs, then cell lipids have been found to be <7% of dry weight [8]. Exceptions exist, however; for instance, the weight of lipid produced by a strain of *Saccharomyces cerevisiae* was 32% of dried cell and palmitoleic acid represented 52% of total FAs [9].

The large-scale production of palmitoleic acid is at present fraught by a number of problems including optimization of cultivation conditions, biomass processing, and analysis of produced lipids. The testing and optimization of cultivation processes will require analytical procedures allowing the analysis of tens or hundreds of samples.

Previous studies aimed mostly at analyzing the overall FAs composition [10] and the composition of FAs in individual lipid classes [11] and showed a considerable variability in both qualitative and quantitative FA composition. For the analysis of unsaturated TAGs having more double bonds, i.e. containing polyunsaturated FAs, GC is less suitable than nonaqueous (NA) RP-HPLC. Some papers describing TAGs from yeast [12, 13] identified only the sum of carbon atoms and the sum of double bonds in all three acyl chains, while others [14–16] succeeded in determining the structure of individual acyls.

The number of molecular species of TAG in natural samples is n^3 (where n is the number of FAs in TAG) when taking into account both regioisomers and enantiomers. NARP-LC represents one of the most common methods for the separation of natural TAGs. It was used to separate hundreds of TAGs [17–19], which were then identified by MS. Silver-ion chromatography is another option for separating TAGs [19]. Stereospecific analysis of individual molecular species of TAGs is relatively difficult. Atmospheric pressure chemical ionization (APCI) MS makes it possible to distinguish regioisomers of TAGs based on the lower relative abundance of fragment ion $[M+H-R_2COOH]^+$, which arises by the neutral loss of an FA from the *sn*-2 position [20–22]. TAG enantiomers (e.g. dipalmitoleoolein (*sn*-PoPoO) and *sn*-OPoPo) having the same FAs in *sn*-1 and *sn*-3 positions provide the same mass spectra and this method is thus unsuitable for distinguishing them.

Using chiral LC on a polysaccharide-based chiral stationary phase, Iwasaki et al. [23] separated without prior derivatization enantiomers of TAGs with very different acyl groups, e.g. eicosapentaenoyl-dicapryoyl-glycerol (e.g. *sn*-ECC or *sn*-CCE). A similar phase was also used by Lisa and Holcapek [24]. Nagai et al. [25] and Gotoh et al. [26] used recycling LC (six recycles) for the separation of 1,2-dioleoyl-3-palmitoyl-*sn*-glycerol and 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol (*sn*-OOP and/or *sn*-POO) from palm oil. Lisa and Holcapek [24] separated, on two cellulose-tris-(3,5-dimethylphenylcarbamate) columns connected in series and using a gradient of hexane/2-propanol (iPrOH) as mobile phase, TAG enantiomers containing one to eight double bonds with different fatty acyl chain lengths including both synthetic standards and TAGs from two natural mixtures—hazelnut oil and human plasma.

This study describes the identification of regioisomers and enantiomers of TAGs containing predominantly palmitoleic and further FAs in different yeasts using RP and chiral phase LC–MS. We propose a highly detailed assay to analyze yeast TAGs with RP and chiral LC separation. Moreover, separation on the two different phases aids in ready identification, both qualitative and semiquantitative, of regioisomers and enantiomers of TAGs containing palmitoleic acid in the molecule. Our APCI-MS method is well suited for the direct determination of TAG profiles and for comparing the production of lipids after a genetic manipulation or a change in microorganism cultivation conditions.

2 Materials and methods

2.1 Standards

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1,3-Palmitolein-2-olein and triheptadecenoin (17:1-17:1-17:1 or MoMoMo) were purchased from Larodan Fine Chemicals AB (Sweden).

TAG enantiomers were prepared according to Lisa and Holcapek [24] and Fraser et al. [27]. Briefly, 13.2 mg (0.1 mmol) of 1,2- or 2,3-isopropylidene-*sn*-glycerol, 0.1 mmol of appropriate FA, 13.4 mg (0.11 mmol) of 4-dimethylaminopyridine and 22.6 mg (0.11 mmol) of *N*,*N*′-dicyclohexylcarbodiimide in 2 mL of dichloromethane were stirred at 20°C for 2 h (Supporting Information Fig. S1). The acetonide was deprotected by the reaction with 0.3 mL of TFA at −20°C for 30 min and the mixture was then neutralized by a cold solution of 2 mol/L sodium hydroxide. The resulting monoacylglycerols were extracted by a mixture of chloroform/methanol (4:1, v/v, 3 mL) and the chloroform layer was evaporated. The monoacylglycerols were stirred with 2.2 mmol of the appropriate FAs, 0.22 mg of 4-dimethylaminopyridine, and 0.24 mmol of *N*, *N*′-dicyclohexylcarbodiimide in 2 mL of dichloromethane for 1.5 h at 20°C. The enantiomers were extracted from the reaction mixture using hexane, evaporated, and injected in hexane solution to the LC–MS (Fig. 1).

2.2 Cultivation

Candida sp. DS 39, *Candida* sp. DS 15, *Kluyveromyces lactis* 2150, *Kluyveromyces marxianus* 41, *Kluyveromyces polysporus*, *Rhodotorula glutinis* 2185, *S. cerevisiae* 2101, *S. cerevisiae* 89, *Torulospora delbrueckii* 39, *Trichosporon cutaneum*, *Yarrowia lipolytica* 2181 were purchased from the Collection of Yeasts and Industrial Microorganisms (Institute of Chemical Technology, Prague). The yeasts were stored at 4°C on solid

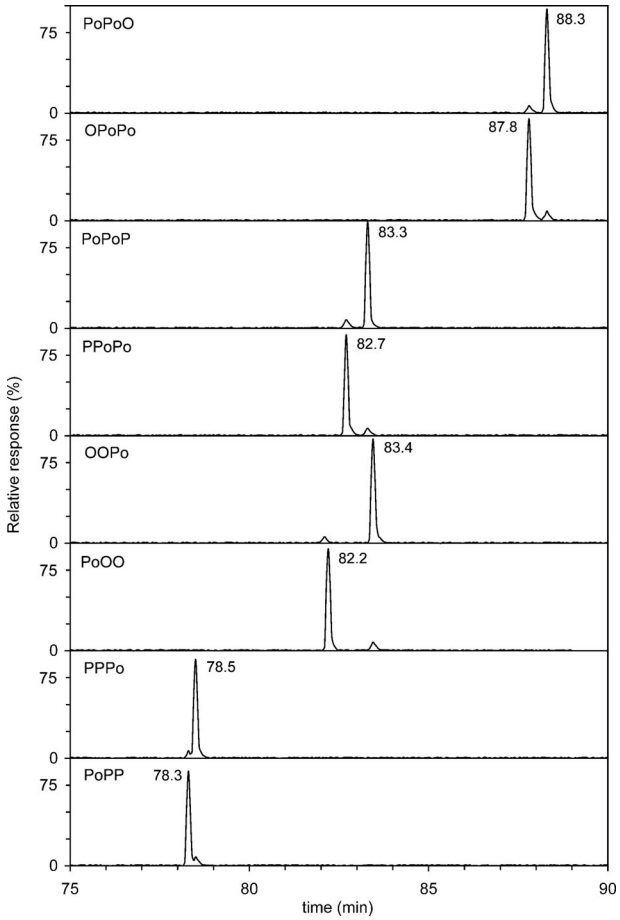

Figure 1. Chiral LC–APCI-MS chromatogram (TIC) of the synthesized (*sn*-PoPP, *sn*-PPPo, *sn*-PoOO, *sn*-OOPo, *sn*-PPoPo, *sn*-PoPoP, *sn*-OPoPo, and *sn*-PoPoO) TAGs. The TAGs(~1 μg/μL in methanol) were chromatographed on two columns (5 μm, 25 cm × 4.6 mm) with 3,5-dimethylphenyl carbamate modified β-cyclodextrin as chiral phase connected in series. The mobile phase was a gradient from 90% of A and 10% of B at 0 min to 50% of A and 50% of B over 120 min, where A is hexane and B is hexane/iPrOH (99:2, v/v). The flow rate, column temperature, and injection volume were 0.5 mL/min, 25°C, and 10 μL, respectively. An API 4000 mass spectrometer was used for identification, for full experimental conditions see the text.

medium (5% malt extract agar) and subcultured biweekly. For long-term storage, stock cultures were maintained in 20% glycerol at −60°C.

Yeast biomass was prepared by cultivating the yeast under aerobic conditions to the early stationary phase (26 h) at 28°C in yeast extract/peptone/dextrose medium (Oxoid, Hampshire, UK) containing 2% peptone (Oxoid) and 2% glucose (Merck, Darmstadt, Germany) per liter of deionized water. The medium was sterilized at 121°C for 20 min before inoculation. The final biomass concentration after inoculation was $OD_{600\,nm} = 0.1$. Inoculum was prepared under aerobic conditions (48 h, yeast extract/peptone/dextrose medium, 28°C).

2.3 Lipid extraction and isolation of TAGs

A 10 mL volume of yeast suspension of 15–17% dry weight was mixed with 2 mL of 0.1 mol/L Na_2CO_3 and the mixture was briefly ground with ballotini glass beads (diameter 0.2 mm) in a mortar, overlaid with liquid nitrogen and ground again. This process was repeated three times, and 50 mL of 0.1 mol/L Na_2CO_3 was finally added. The crushed yeast was extracted with a chloroform/methanol mixture according to Bligh and Dyer [28]. The sample was centrifuged, and the lower phase was evaporated to dryness and analyzed by MS.

Total lipid extracts for further analysis of FAs were applied to Sep-Pak Vac Silica cartridge 35 cc (Waters, Milford, MA, USA; with 10 g of silica normal-phase), and TAGs were subsequently eluted from the cartridge with 40 mL of chloroform/methanol (9:1). The eluate containing TAGs was evaporated.

2.4 FA methyl ester analysis

The total TAGs (~5 mg) were saponified overnight in 10% KOH/MeOH at room temperature. An FA fraction obtained from the saponification was partitioned between alkali solution (pH 9) and diethyl ether to remove basic and neutral components. The aqueous phase containing FAs was acidified to pH 2 and extracted with hexane. The FA fraction was methylated using BF_3/MeOH (14% solution of BF_3 from Sigma–Aldrich).

GC–MS analysis of the FA methyl esters (FAMEs) was done on a GC–MS system consisting of a Varian 450-GC (Varian, Middelburg, the Netherlands), a Varian 240-MS ion trap detector with electron ionization, and a CombiPal autosampler (CTC, USA) equipped with a split/splitless injector. A DB-5MS column was used for separation (60 m, 0.25 mm id, 0.25 μm film thickness). The temperature program started at 60°C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 160°C at a rate of 25°C/min. The second temperature ramp was up to 280°C at a rate of 2.5°C/min, this temperature being maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280°C. Mass spectra were recorded at 3 scans/s under electron ionization at 70 eV, mass range m/z 50–450. FAMEs were identified according to their mass spectra [29, 30] and using a mixture of chemical standards obtained from Sigma–Aldrich.

2.5 NARP-LC–APCI-MS

The LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent Technologies, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm id, 5 μm particle size (Hichrom, Berkshire, UK), in series. This setup represented a high-efficiency column, approximately 52 000 plates (104 plates/1 mm), and was operated at a flow rate of

1 mL/min, injection volume of 10 μL, and column temperature of 25°C. The TAGs were separated using a gradient solvent program with acyl carbon number (ACN) and iPrOH) as follows: initial ACN/iPrOH (99:1, v/v); linear from 5 to 110 min ACN/iPrOH 30:70 v/v; held until 10 min; the composition was returned to the initial conditions over 10 min. The performance was measured by triheptadecenoin as an internal standard under the conditions given above.

The detector was an AB Sciex API 4000 mass spectrometer (AB Sciex, Ontario, Canada) using positive ion APCI mode with the following settings: the ionization mode was positive, source temperature 420°C, nebulizer gas (GS1) 5, nebulizer current 3 μA, curtain gas 10, collision gas and declustering potential 100 V. The optimal collision energy was dependent on the type of experiment and was set to +10 V (full scan mode) or +30 V (product ion mode). The full scan spectra were obtained at m/z 200–1100. To identify the exact composition of the high m/z value TAG species, an enhanced product ion spectrum (optimal CE +35 V) was scanned for all ions with an m/z value over 800.

Collision-induced dissociation ion mass spectra were acquired by colliding the Q1-selected precursor ions with Ar gas as a collision target gas and applying a collision energy of 50 eV in Q2. The scanning range of Q3 was m/z 200–1100 with a step size of m/z 0.3 and a dwell time of 1 ms. A peak threshold of 0.3% intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.2 software.

2.6 Chiral LC–APCI-MS

The LC system used for separation in the chiral mode was the same as that used in the RP mode. TAGs (~1 μg/μL in methanol) were chromatographed on two Astec CYCLOBOND™ I 2000 DMP (3,5-dimethylphenyl carbamate modified β-cyclodextrin) chiral LC columns connected in series, 5 μm, 25 cm × 4.6 mm from Sigma–Aldrich. The mobile phase was a gradient from 90% of A and 10% of B at 0 min to 50% of A and 50% of B over 120 min, where A is hexane and B is hexane/iPrOH (99:2, v/v) mixture [24]. The flow rate, column temperature, and injection volume were 0.5 mL/min, 25°C, and 10 μL, respectively. Other separation conditions used were as in the NARP-LC.

3 Results and discussion

3.1 NARP-LC–APCI-MS

TAGs are most often separated by using a nonaqueous mobile phase with octadecyl silica gel columns, i.e. NARP-LC [18, 31] or by silver-ion chromatography [19]. When using NARP-LC, the elution order depends on the length of the acyl chains and the number of double bonds [32]; this has allowed for the separation of hundreds of different TAGs in different plant and/or animal oils and fats [18].

TAGs often occur as regioisomers, i.e. positional isomers on the glycerol backbone [33]. Lin et al. [34] have described the separation of *sn*-POP with retention time 33.12 min and *sn*-PPO with 33.16 min. Similar results were obtained by Momchilova et al. [35], who obtained a nearly complete separation of *sn*-PPO from *sn*-POP with a resolution of 0.9. As described by Rezanka et al. [36, 37] and Schreiberova et al. [22], the separation of regioisomeric TAGs as intact species by NARP-LC is possible and it was found that symmetrical TAGs elute before the corresponding asymmetrical TAGs. The mass spectrum of the $[M+H]^+$ ions of TAGs yielded abundant fragment ions corresponding to $[M+H-RCOOH]^+$, i.e. ions commonly denoted as $[DAG]^+$. Their abundance obeys the rule [21, 38, 39] about the energetically less favorable loss of FA from position *sn*-2 and formation of $[M-R_2COO]^+$, i.e. 1,3-$[DAG]^+$ ions, which was applied also to our TAGs. This implies that the different abundance or the ratio of the 1,3- or 1,2- (2,3-) $[DAG]^+$ ions makes it possible to determine if the given acid esterifies the primary or the secondary hydroxyls of glycerol. An example is the MS/MS spectrum of one of abundant TAGs, i.e. oleopalmitoleoolein (*sn*-OPoO; for abbreviations of FAs, see Table 1; Supporting Information Fig. S2), which contains two different FA and exhibits, in addition to $[M+H]^+$ at m/z 857, also further ions. The FAs in *sn*-OPoO were also determined by fragment ions in the tandem spectra of TAG—see the values $[RCO + 74]^+$ at m/z 339 $[O + 74]^+$, and m/z 311 $[Po + 74]^+$, $[RCO]^+$ at m/z 265 $[O]^+$, and m/z 237 $[Po]^+$ for oleic and palmitoleic acid, respectively. The least abundant $[M+H-RCOO]^+$ ion ($[DAG]^+$ ion, i.e. $[OO]^+$ at m/z 603), is formed by the loss of palmitoleic acid from the *sn*-2 position. The two more abundant $[DAG]^+$ ions are due to loss of palmitoleate from the *sn*-1 or *sn*-3 position giving $[PoO]^+$ ion at m/z 575. The $[OO]^+/[OPo]^+$ ratio observed for *sn*-OPoO (Supporting Information Fig. S2) is only 32:100. Consequently, the two regioisomers (*sn*-OPoO and *sn*-PoOO, or its enantiomer *sn*-OOPo) show two different spectra (Supporting Information Figs. S2–S4). Further TAGs were identified in a similar manner.

The use of 17:1-17:1-17:1 TAG (having odd numbers of FA) as internal standard has several advantages: this TAG is commercially available and does not have to be synthesized; its occurrence in eukaryotic organisms has not yet been described, and if it was present, then in amounts so low that its presence can be neglected. This was confirmed by the results of FAMEs analysis by GC–MS, which did not reveal the presence of the corresponding 17:1 acid (the threshold value for integration parameters was 0.1% total FAs).

3.2 Chiral LC

Figure 2 presents the chiral LC–APCI-MS analysis of commercially obtained saturated, two unsaturated (PoPoPo and OOO), and several synthesized standards of TAGs. The retention time of the TAGs depends on the number of double bonds and increases with increasing number of double

Table 1. FA content in yeast strains under study, identified by means of GC–MS of FAMEs

Strain	9-16:1 (Po)	16:0 (P)	9,12-18:2 (L)	9-18:1 (O)	18:0 (S)	20:0 (A)	Dry weight (g/L)	Lipids in percentage of dry weight	Lipids (g/L)	mg Po/1 L*
Kluyveromyces polysporus	59.2	7.4	2.7	28.9	1.0	0.8	4.51	5.11	0.23	129
K.luyveromyces lactis 2150	28.7	11.1	18.3	38.5	1.5	1.9	4.87	4.37	0.21	57
Kluyveromyces marxianus 41	16.1	19.7	29.1	30.0	2.2	2.9	4.74	4.53	0.21	32
Saccharomyces cerevisiae 89	38.8	12.8	3.4	37.9	3.4	3.7	3.88	4.17	0.16	59
Saccharomyces cerevisiae 2101	39.3	11.4	3.5	38.1	4.1	3.6	4.16	3.81	0.16	60
Torulospora delbrueckii 39	22.6	25.0	15.8	27.4	2.6	6.6	4.46	4.64	0.21	45
Yarrowia lipolytica 2181	14.0	13.3	18.1	49.0	1.1	4.5	2.40	17.90	0.43	57
Candida sp. 39	8.8	16.7	29.7	38.6	2.3	3.9	4.46	35.40	1.58	132
Candida sp. 15	3.4	16.0	19.7	52.8	4.9	3.2	5.66	37.10	2.10	68
Rhodotorula glutinis 2185	0.8	12.4	12.9	65.4	7.1	1.4	3.00	33.20	1.00	2
Trichosporon cutaneum	2.6	17.0	48.8	28.1	1.8	1.7	1.34	48.90	0.66	16

*mg per 1 L culture broth.

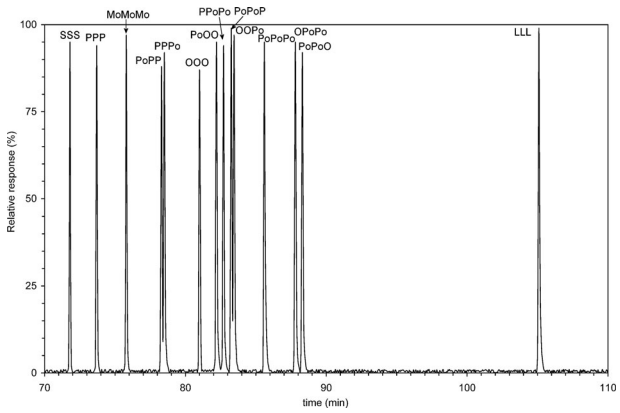

Figure 2. Chiral LC–APCI-MS chromatogram (TIC) of the commercially obtained standards of TAGs. For experimental conditions see Fig. 1 and the text.

bonds in the acyl chains, decreasing, on the other hand, with increasing acyl chain lengths.

The column packed with chiral oligosaccharide β-cyclodextrin modified by tris-(3,5-dimethylphenylcarbamate) was used for the separation of enantiomers of TAGs. Since it is in fact a separation on a normal phase, the elution was done with a nonpolar mixture of solvents, i.e. hexane/iPrOH. As already described and optimized by Lisa and Holcapek [24], the most suitable was assumed to be the gradient of hexane and hexane/iPrOH (99:1, v/v). As our stationary phase is not based on cellulose but on β-cyclodextrin, we had to modify the gradient and the content of the polar solvent iPrOH, see Section 2. Thanks to this we succeeded in separating enantiomers of TAGs (*sn*-PPL/*sn*-LPP or *sn*-LLP/*sn*-PLL, etc.) that were not previously separated [24].

In contrast to the number of double bonds in FAs esterifying the primary hydroxyl of glycerol, the number of double bonds in FAs esterifying the secondary hydroxyl group has a negligible effect. If the FA with double bond(s) is in the *sn*-1 position, then this enantiomer has a higher retention time, see, e.g. *sn*-PPO (75.4 min) and *sn*-OPP (75.9 min), Table 2. The effect of the number of double bonds in *sn*-2 acyl is negligible, see, e.g. two pairs of TAGs differing only in unsaturation in *sn*-2 acyl, i.e. *sn*-POO (78.8 min) and *sn*-OOP (80.1 min) versus *sn*-PLO (83.8 min) and *sn*-OLP (84.9 min); the difference is 1.3 and 1.1 min, respectively. Most enantiomers were separated including those that have combinations of saturated and diunsaturated (linoleic acid) FAs in *sn*-1 and *sn*-3 positions, i.e. the pairs *sn*-LPP/*sn*-PPL or *sn*-LLP/*sn*-PLL. This finding can be explained by the fact that the stationary phase used in this study, i.e. β-cyclodextrin-tris(3,5-dimethylphenylcarbamate), differed from that used by Lisa and Holcapek [24], i.e. cellulose-tris-(3,5-dimethylphenylcarbamate). TAGs having three different FAs and forming thus six molecular species are too complex to be separated by using this system and their elution properties cannot be determined without the aid of authentic standards of individual enantiomers. The retention times of two groups of six TAGs, i.e. *sn*-PLO and *sn*-SLO, were determined only from the data of Lisa [24].

Only negligible differences in mass spectra were found in the enantiomers (see Supporting Information Figs. S3 and S4). The spectra therefore cannot be used for their identification and the only way of identification is their separation with subsequent identification by means of retention times of standards, which were prepared according to previous reports [24, 27], see Supporting Information Fig. S3 and Fig. 1, which presents the chromatograms of TAGs, shows that the optical purity of individual enantiomers was about 95%.

3.3 NARP-LC of natural samples

The total lipid extracts of the 11 yeast strains were separated by NARP-LC, see Fig. 3 for analysis of *K. polysporus*. By using MS/MS we identified 44 molecular species of TAGs

Table 2. Relative quantities[a)] (%) of molecular species of TAGs identified in yeasts, their retention times, equivalent carbon number, number of double bond(s), calculated *m/z* of $[DAG]^+$ and $[M+H]^+$

Rt (Ch*)	Rt (RP)	TAG**	ECN[b)]	ACN	DB	$[M+H]^+$	DAG 1.2	DAG 2.3	DAG 1.3	*K. p.*	*K. l.*	*K. m.*	*S. c.* 89	*S. c.* 2101	*T. d.*	*Y. l.*	*C.* sp. 39	*C.* sp. 15	*R. g.*	*T. c.*
71.2	104.1	APP	52	52	0	863.81	607.57	551.50	607.57	0.0	0.1	0.4	0.2	0.1	1.2	0.2	0.3	0.2	0.1	0.1
71.4	104.2	SSP	52	52	0	863.81	607.57	579.54	579.54	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.0
71.8	109.1	SSS	54	54	0	891.84	607.57			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0
72.1	96.8	PoSS	50	52	1	861.79	577.52	607.57	577.52	0.0	0.0	0.0	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.0
72.4	102.0	AOP	52	54	1	889.82	633.58	577.52	607.57	0.0	0.3	0.6	0.5	0.5	1.3	0.8	0.9	0.7	0.3	0.2
72.6	98.7	PSP	50	50	0	835.78	579.54	579.54	551.50	0.0	0.2	0.1	0.0	0.0	0.3	0.0	0.2	0.3	0.2	0.1
72.8	107.1	AOS	54	56	1	917.85	633.58	605.55	635.60	0.0	0.0	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.2	0.0
73.0	102.3	SSO	52	54	1	889.82	607.57	605.55	605.55	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.9	0.0
73.7	93.2	PPP	48	48	0	807.74	551.50			0.1	0.4	2.4	0.6	0.4	4.6	2.7	1.5	1.1	0.4	1.3
73.8	98.9	SPP	50	50	0	835.78	579.54	551.50	579.54	0.0	0.1	0.2	0.2	0.2	0.2	0.1	0.0	0.0	0.0	0.0
74.2	96.8	SPO	50	52	1	861.79	579.54	577.52	605.55	0.0	0.0	0.3	0.0	0.7	0.3	0.1	0.3	0.6	0.9	0.2
74.8	96.5	AOPo	50	54	2	887.81	633.58	575.50	605.55	0.3	0.7	0.5	1.6	1.6	1.2	0.9	0.4	0.2	0.0	0.0
75.1	96.8	SOP	50	52	1	861.79	605.55	577.52	579.54	0.1	0.0	0.3	0.1	0.2	0.2	0.2	0.2	0.5	0.3	0.1
75.2	102.1	SOS	52	54	1	889.82	605.55	605.55	607.57	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.2	1.5	0.0
75.2	102.3	OSS	52	54	1	889.82	605.55	607.57	605.55	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.3	0.0
75.4	91.5	PPO	48	50	1	833.76	551.50	577.52	577.52	0.5	0.4	3.0	0.4	0.2	3.9	1.9	0.4	0.9	0.5	0.5
75.5	100.5	AOO	52	56	2	915.84	633.58	603.54	633.58	0.0	0.1	0.9	0.1	0.1	1.0	2.3	0.4	0.3	0.3	0.1
75.8	100.2	MoMoMo	51	51	0	849.76	579.54			–	–	–	–	–	–	–	–	–	–	–
75.9	91.3	POP	46	50	2	833.76	577.52	577.52	551.50	0.3	0.2	0.3	0.2	0.2	0.4	0.3	2.4	1.8	1.1	1.3
75.9	91.5	OPP	48	50	1	833.76	577.52	551.50	577.52	7.3	1.1	0.6	1.2	1.0	0.5	0.2	0.5	0.6	0.6	0.4
76.1	100.3	OAO	52	56	2	915.84	633.58	633.58	603.54	0.1	0.4	0.0	0.2	0.1	0.2	0.3	1.2	1.7	0.6	0.2
76.5	95.3	SOO	50	54	2	887.81	605.55	603.54	605.55	0.0	0.0	0.3	0.1	0.0	0.5	0.3	0.0	0.0	0.1	0.0
76.9	91.4	PPoS	48	50	1	833.76	549.49	577.52	579.54	0.1	0.2	0.2	0.5	0.5	0.4	0.1	0.1	0.1	0.0	0.0
77.1	100.5	OOA	52	56	2	915.84	603.54	633.58	633.58	0.1	0.5	0.1	0.9	1.3	0.1	0.6	0.3	0.4	0.4	0.1
77.2	95.1	OSO	50	54	2	887.81	605.55	605.55	603.54	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.1	1.0	0.1
77.3	96.3	ALP	50	54	2	887.81	631.57	575.50	607.57	0.0	0.1	0.6	0.1	0.0	0.8	0.3	0.7	0.3	0.1	0.4
78.1	85.5	PPoP	46	48	1	805.73	549.49	549.49	551.50	0.3	0.0	0.3	0.1	0.1	0.3	0.1	0.5	0.2	0.0	0.2
78.3	85.7	PoPP	46	48	1	805.73	549.49	551.50	549.49	2.4	0.9	0.3	1.5	1.2	0.2	0.1	0.1	0.0	0.0	0.0
78.5	85.7	PPPo	46	48	1	805.73	551.50	549.49	549.49	0.2	0.1	1.6	0.2	0.2	3.8	0.5	0.2	0.0	0.0	0.0
78.7	95.3	OOS	50	54	2	887.81	603.54	605.55	605.55	0.1	0.2	0.1	0.3	0.2	0.0	0.0	0.0	0.0	0.2	0.0
78.8	89.9	POO	48	52	2	859.78	577.52	603.54	577.52	0.3	0.4	4.1	0.1	0.0	5.1	7.2	1.4	2.4	2.2	1.0
79.1	84.8	SPoPo	46	50	2	831.74	577.52	547.47	577.52	0.1	0.0	0.1	0.1	0.2	0.2	0.1	0.0	0.0	0.0	0.0
79.2	84.8	PoPoS	46	50	2	831.74	547.47	577.52	577.52	0.7	0.4	0.1	1.4	1.3	0.1	0.0	0.0	0.0	0.0	0.0
79.3	89.7	OPO	48	52	2	859.78	577.52	577.52	603.54	0.2	0.3	0.3	0.2	0.3	0.3	0.3	4.6	4.7	5.6	2.1
79.4	84.6	PoSPo	46	50	2	831.74	577.52	577.52	547.47	0.1	0.0	0.0	0.2	0.3	0.0	0.0	0.1	0.0	0.0	0.0
80.0	84.3	OPPo	46	50	2	831.74	577.52	549.49	575.50	1.7	0.7	1.2	2.8	3.1	2.9	1.3	1.1	0.5	0.1	0.1
80.1	89.9	OOP	48	52	2	859.78	603.54	577.52	577.52	5.8	4.9	0.9	4.7	4.0	0.6	1.2	2.1	1.8	4.2	0.7
80.2	90.7	SOPo	48	52	2	859.78	605.55	575.50	577.52	0.6	0.5	0.4	1.9	1.8	0.5	0.2	0.3	0.2	0.0	0.0
80.4	101.4	ALS	52	56	2	915.84	631.57	603.54	635.60	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.1
80.7	96.2	SLS	50	54	2	887.81	603.54	603.54	607.57	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.8	0.1

Table 2. Continued

Rt (Ch*)	Rt (RP)	TAG**	ECN[b)]	ACN	DB	$[M+H]^+$	DAG 1.2	DAG 2.3	DAG 1.3	*K. p.*	*K. l.*	*K. m.*	*S. c.* 89	*S. c.* 2101	*T. d.*	*Y. l.*	*C.* sp. 39	*C.* sp. 15	*R. g.*	*T. c.*
80.7	96.4	SSL	50	54	2	887.81	607.57	603.54	603.54	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0
80.8	84.2	OPoP	46	50	2	831.74	575.50	549.49	577.52	1.8	2.1	1.3	2.4	1.8	3.3	1.4	0.8	0.3	0.0	0.2
81.0	88.5	OOO	48	54	3	885.79	603.54			5.2	12.4	8.1	15.8	15.7	6.1	22.6	19.1	28.0	37.6	12.0
81.3	85.2	PLP	46	50	2	831.74	575.50	575.50	551.50	0.0	0.0	0.2	0.0	0.1	0.2	0.0	2.2	0.8	0.2	2.1
81.6	96.4	LSS	50	54	2	887.81	603.54	607.57	603.54	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
82.1	94.9	ALO	50	56	3	913.82	631.57	601.52	633.58	0.0	0.4	0.9	0.1	0.1	0.8	1.1	1.5	0.9	0.3	0.6
82.2	82.8	PoOO	46	52	3	857.76	575.50	603.54	575.50	12.4	10.2	1.3	13.9	12.5	1.7	0.8	1.1	1.1	0.0	0.1
82.5	85.4	LPP	46	50	2	831.74	575.50	551.50	575.50	0.0	0.6	0.2	0.2	0.0	0.1	0.0	0.3	0.2	0.1	0.8
82.6	82.6	OPoO	46	52	3	857.76	575.50	575.50	603.54	0.7	0.9	1.1	1.4	1.6	0.7	0.9	3.1	4.2	0.2	0.3
82.6	89.6	SLO	48	54	3	885.79	603.54	601.52	605.55	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.1	0.2	0.1	0.2
82.7	78.6	PPoPo	44	48	2	803.71	549.49	547.47	549.49	0.3	0.4	5.8	0.4	0.8	3.2	0.5	0.0	0.0	0.0	0.0
82.8	85.4	PPL	46	50	2	831.74	551.50	575.50	575.50	0.0	0.1	3.2	0.0	0.0	2.5	0.9	0.2	0.3	0.1	0.6
82.9	78.5	PoPPo	44	48	2	803.71	549.49	549.49	547.47	0.6	0.5	0.3	0.7	0.5	0.3	0.1	0.4	0.1	0.0	0.0
83.3	78.6	PoPoP	44	48	2	803.71	547.47	549.49	549.49	4.1	1.6	1.9	4.2	3.9	0.7	0.2	0.0	0.0	0.0	0.0
83.4	82.8	OOPo	46	52	3	857.76	603.54	575.50	575.50	1.3	1.9	6.3	1.0	2.3	12.4	9.9	0.4	0.5	0.0	0.1
83.7	91.1	SLP	48	52	2	859.78	603.54	575.50	579.54	0.0	0.1	0.4	0.0	0.1	0.3	0.1	0.4	0.4	0.2	0.4
83.8	83.8	PLO	46	52	3	857.76	575.50	601.52	577.52	0.0	1.2	0.4	0.0	0.1	0.2	0.0	0.5	0.6	0.8	2.1
84.1	89.6	LSO	48	54	3	885.79	603.54	605.55	601.52	0.0	0.2	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.4	0.3
84.6	89.6	SOL	48	54	3	885.79	605.55	601.52	603.54	0.0	0.1	0.3	0.1	0.1	0.0	0.1	0.3	0.7	0.2	0.3
84.8	89.6	OLS	48	54	3	885.79	601.52	603.54	605.55	0.0	0.0	0.2	0.0	0.0	0.1	0.1	0.2	0.0	0.2	0.0
84.9	83.8	OLP	46	52	3	857.76	601.52	575.50	577.52	0.1	0.2	1.3	0.0	0.0	0.2	0.3	1.3	0.7	0.4	1.3
85.2	83.8	LOP	46	52	3	857.76	601.52	577.52	575.50	0	0.3	1.9	0.2	0.3	0.5	1	0.5	2.4	4.8	4.5
85.3	89.6	OSL	48	54	3	885.79	605.55	603.54	601.52	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.2	0.1	0.1
85.6	70.0	PoPoPo	42	48	3	801.70	547.47			26.1	7.2	1.3	17.0	17.3	4.4	2.8	0.2	0.0	0.0	0.0
85.8	89.6	LOS	48	54	3	885.79	601.52	605.55	603.54	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.0
86.1	78.0	PLPo	44	50	3	829.73	575.50	573.49	549.49	0.3	1.8	3.0	0.5	0.5	2.7	2.9	1.4	0.3	0.0	0.6
86.3	83.8	OPL	46	52	3	857.76	577.52	575.50	601.52	0.0	0.7	1.3	0.2	0.0	0.4	0.4	0.8	0.8	3.2	1.7
86.4	77.1	PoOPo	44	50	3	829.73	575.50	575.50	547.47	6.3	4.6	0.6	2.3	2.8	1.0	0.3	0.5	0.2	0.0	0.1
87.6	82.2	OLO	46	54	4	883.78	601.52	601.52	603.54	0.1	1.7	0.7	0.3	0.3	0.4	1.7	3.0	9.4	7.0	4.9
87.8	77.3	OPoPo	44	50	3	829.73	575.50	547.47	575.50	1.4	0.7	1.7	0.9	1.1	8.4	4.1	0.3	0.0	0.0	0.0
88.2	82.4	LOO	46	54	4	883.78	601.52	603.54	601.52	0.3	6.1	0.9	0.8	0.9	0.3	2.5	0.2	3.1	2.4	3.1
88.3	77.3	PoPoO	44	50	3	829.73	547.47	575.50	575.50	14.2	9.2	0.3	13.3	12.8	0.9	0.4	0.2	0.0	0.0	0.0
89.7	82.4	OOL	46	54	4	883.78	603.54	601.52	601.52	0.1	0.8	5.6	0.3	0.3	2.6	8.7	1.8	1.9	3.0	1.9
90.1	89.3	ALL	48	56	4	911.81	631.57	599.50	631.57	0.0	0.2	0.8	0.0	0.0	0.6	0.4	1.2	0.3	0.1	1.1
91.0	83.5	SLL	46	54	4	883.78	603.54	599.50	603.54	0.0	0.0	0.5	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1
91.8	83.5	LLS	46	54	4	883.78	599.50	603.54	603.54	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.3
92.0	77.6	LLP	44	52	4	855.74	599.50	575.50	575.50	0.0	0.7	0.4	0.0	0.0	1.3	0.9	1.6	0.6	0.1	1.3
92.1	77.6	PLL	44	52	4	855.74	575.50	599.50	575.50	0.0	0.2	4.2	0.0	0.0	0.2	0.1	1.4	0.2	0.2	2.2
92.7	83.3	LSL	46	54	4	883.78	603.54	603.54	599.50	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.3	0.2	0.2	0.7
93.7	76.7	OLPo	44	52	4	855.74	601.52	573.49	575.50	1.0	6.1	4.5	1.5	1.5	2.9	3.5	3.4	1.0	0.1	0.9
93.9	77.4	LPL	44	52	4	855.74	575.50	575.50	599.50	0.0	0.2	0.7	0.0	0.0	0.2	0.2	2.8	1.0	0.2	6.5

Table 2. Continued

Rt ([Cn*])	Rt (RP)	TAG**	ECN[b]	ACN	DB	$[M+H]^+$	DAG 1.2	DAG 2.3	DAG 1.3	*K. p.*	*K. l.*	*K. m.*	*S. c.* 89	*S. c.* 2101	*T. d.*	*Y. l.*	*C.* sp. 39	*C.* sp. 15	*R. g.*	*T. c.*
95.2	76.2	LLO	44	54	5	881.76	599.50	601.52	601.52	0.0	0.4	3.5	0.1	0.0	1.0	1.9	2.1	2.3	3.2	4.8
96.9	76.2	OLL	44	54	5	881.76	601.52	599.50	601.52	0.1	2.8	3.4	0.1	0.1	0.8	1.7	2.6	2.7	3.5	5.6
97.3	70.6	PoLPo	42	50	4	827.71	573.49	573.49	547.47	0.6	1.3	0.2	0.5	0.4	0.2	0.1	1.1	0.1	0.0	0.1
97.5	76.0	LOL	44	54	5	881.76	601.52	601.52	599.50	0.0	0.7	1.3	0.0	0.1	0.2	0.8	7.6	5.3	6.0	7.2
98.4	70.8	LPoPo	42	50	4	827.71	573.49	547.47	573.49	0.1	0.8	1.8	0.2	0.3	2.1	0.7	0.5	0.0	0.0	0.0
99.5	70.8	PoPoL	42	50	4	827.71	547.47	573.49	573.49	1.4	2.5	0.3	0.8	0.9	0.1	0.2	0.7	0.0	0.0	0.0
101.1	70.2	LLPo	42	52	5	853.73	599.50	573.49	573.49	0.0	0.4	2.4	0.0	0.0	1.0	0.8	1.5	0.4	0.0	0.6
101.8	70.2	PoLL	42	52	5	853.73	573.49	599.50	573.49	0.1	1.9	1.1	0.1	0.2	0.4	0.2	0.8	0.2	0.0	0.4
102.4	70.0	LPoL	42	52	5	853.73	573.49	573.49	599.50	0.0	0.5	0.9	0.0	0.0	0.3	0.3	1.6	0.6	0.0	1.0
105.1	69.8	LLL	42	54	6	879.74	599.50			0.0	1.9	2.0	0.0	0.0	2.2	1.7	8.8	6.9	0.5	19.5

K. p., Kluyveromyces polysporus; *K. l., Kluyveromyces lactis* 2150; *K. m., Kluyveromyces marxianus* 41; *S. c.* 89, *Saccharomyces cerevisiae* 89; *S. c.* 2101, *Saccharomyces cerevisiae* 2101; *T. d., Torulospora delbrueckii* 39; *Y. l., Yarrowia lipolytica* 2181; *C.* sp. 39, *Candida* sp. 39; *C.* sp. 15, *Candida* sp. 15; *R. g., Rhodotorula glutinis* 2185; *T. c., Trichosporon cutaneum*; ECN, equivalent carbon number; ACN, acyl carbon number; Rt, retention times.

a) The values given in columns *K. p., K. l., K. m.*, etc. are area ratios of TIC chromatograms and indicate approximate ratios of TAG.

b) ECN = ACN − 2 × DB). *Chiral retention time; **Abbreviations of trivial FA names A, arachidic acid; L, linoleic acid; Mo, margaroleic acid; O, oleic acid; P, palmitic acid; Po, palmitoleic acid; S, stearic acid.

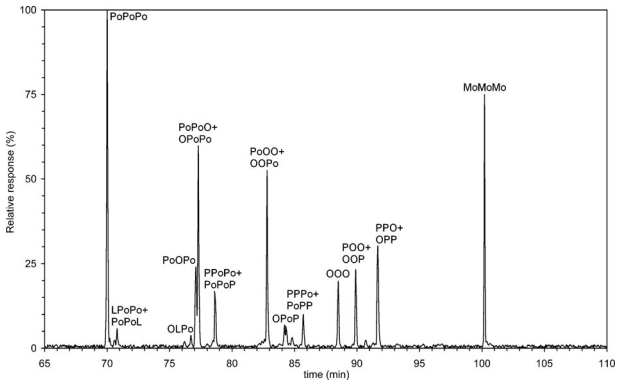

Figure 3. NARP-LC–APCI-MS chromatogram (TIC) of the TAGs from the yeast *K. polysporus*. Separation was performed on two Hichrom columns HIRPB-250AM 250 × 2.1 mm id, 5-um particle size, connected in series, with a flow rate of 1 mL/min, injection volume of 10 μL, and column temperature of 25°C. TAGs were separated using a gradient solvent program with ACN and iPrOH) as follows: initial ACN/iPrOH (99:1, v/v); linear from 5 to 110 min ACN/iPrOH 30:70, v/v); held until 10 min; the composition was returned to the initial conditions over 10 min. API 4000 mass spectrometer was used for identification, for full experimental conditions see the text.

with abundances >0.02% of total TAGs and having up to 59 carbon atoms in the molecule. MS/MS analysis and identification of $[RCO]^+$ and $[RCO + 74]^+$ type ions [20, 21] showed the presence of six major FAs, i.e. palmitoleic, palmitic, linoleic (9,12-18:2 or L), oleic, stearic, and arachidic (20:0 or A) acids. Since MS analysis produces a large number of scans, we used for simplification the neutral-loss scan NLS method, which made it possible to greatly simplify the analysis of the molecular composition of complex mixtures of yeast TAGs without knowing in advance their FA composition. Supporting Information Fig. S5 depicts three different producers (*K. polysporus, Y. lipolytica*, and *R. glutinis*) of palmitoleic acid based on total ion current (TIC) and NLS of palmitoleic acid, i.e. ($[M+H-C_{15}H_{29}COOH]^+$).

In each MS/MS spectrum, the corresponding neutral loss fragments were identified and the relative intensities of the corresponding TAG species, which were designated by the total number of carbon atoms and double bonds in their three FA moieties, were determined. A total of 94 TAGs with unique molecular formulas were identified in the 11 yeast strains. The MS/MS analysis showed that the molecular composition of the TAGs was relatively heterogeneous, many precursor ions representing up to four isobaric species, which were separated either as regioisomers (by NARP-LC) or as enantiomers (by chiral LC).

The relative proportion given in Table 1 was determined from the abundance of each TIC. The chromatograms of the 11 strains (see Supporting Information Fig. S6) indicate that the strains can be divided into three groups composed of low-, medium-, and high-producing strains according to the abundance of appropriate TAGs, i.e. TAGs containing at least one palmitoleic acid in the molecule. This is also documented by GC–MS analysis of FAMEs obtained from total TAGs

(Table 1), which confirmed the presence of six major FAs, i.e. palmitic, palmitoleic, stearic, oleic, linoleic, and arachidic acid. The content of palmitoleic acid varied between 0 and 60% of total FAs and depended predominantly on the strain selection. The yeasts can be divided into low-, medium-, and high-production strains based on the content of palmitoleic acid. Strains containing palmitoleic acid >25% are taken to be high producers, those containing 10 to 25% total FAs are medium. Strains containing <10% palmitoleic acid are low-production strains. Four strains (*K. polysporus*, *K. lactis* 2150, *S. cerevisiae* 89, and *S. cerevisiae* 2101) form a group; as evident from Table 1, the content of palmitoleic acid did not decrease to <25% of total FAs. Most interesting in this table is the last column, which shows that only two strains are biotechnologically interesting, *K. polysporus* containing nearly 60% palmitoleic acid and the medium-producing strain *Candida* sp. DS 39, which contained <9% palmitoleic acid but the content of its lipids per liter exceeded 1.5 g. The content of palmitoleic acid in both strains is thus 130 mg/L.

Although all oleaginous yeast strains under study produce similar TAGs, there are clear differences in the unsaturation and chain length of FAs, especially in TAGs containing palmitoleic acid. The production of total FAs in yeast lipids or TAGs has been widely studied [40]. By contrast, the composition of TAGs, i.e. identification of molecular species of TAGs in yeast, has received less attention. As opposed to plant and animal oils and fats for which intensive TAG identification has been performed [29], only a few papers have so far dealt with the identification of molecular species of yeast TAGs.

The comparison of the content of TAGs in yeasts other than *Saccharomyces* is difficult due to the paucity of studies describing the analysis of molecular species of TAGs. For instance, Singh and Prasad [41] in their study of *Candida albicans* identified TAGs only by the sum of carbon atoms and the sum of double bonds, not as molecular species; the major TAGs in strain F5, in which the content of TAGs having palmitoleic acid in the molecule was 21% included 50:1, 50:2, 52:3, and 52:4 TAGs. The authors did not address the content of FA in individual TAGs, although the TAGs containing palmitoleic acid are expected to include the following molecular species: PSPo, POPo, PoOO, and PoLO. Similarly, the major TAGs in *Schizosaccharomyces pombe* were 54:3 (probably OOO) and 54:2 (probably OOS or LSS) [13].

3.4 Chiral LC of natural samples

TAG enantiomers have so far been separated and identified by chiral chromatography only as mono- and diacylglycerols. This approach was used to identify three TAGs, i.e. *sn*-OLL, *sn*-LLO, and *sn*-LOL in peanut and cottonseed oils [42]. TAGs from sunflower oil [43] were composed of *sn*-OLL, *sn*-LLO, and *sn*-LOL in a ratio of 24:63:13. Redden et al. [44] also found that the dilinoleoyl-γ-linolenin TAGs from evening primrose oil have γ-1inolenic acid (G) distributed in TAGs (*sn*-LLG, *sn*-GLL, and *sn*-LGL) at a ratio of 19.5, 33.8, and 47.2%.

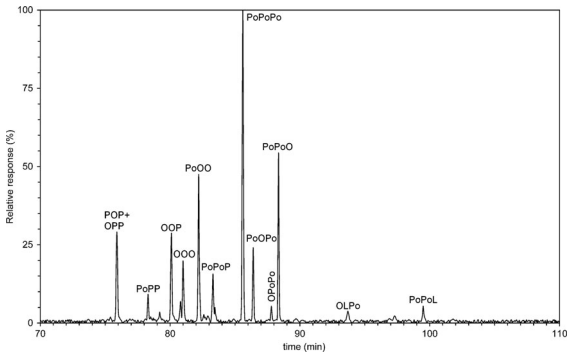

Figure 4. Chiral LC–APCI-MS chromatogram (TIC) of the TAGs from the yeast *K. polysporus*. For experimental conditions see Fig. 1 and the text.

The change in the center of chirality on carbon *sn*-2 and thus the formation of both enantiomers could take place by intramolecular transesterification—see, e.g. the *in vitro* experiment of Iwasaki et al. [23], in which interesterification of tricapryloylglycerol with ethyleicosapentaenoate under catalysis by *Rhizomucor miehei* lipase gave rise to an asymmetrical pair of TAGs, i.e. 1-eicosapentaenoyl-2,3-dicapryroyl-*sn*-glycerol and 1,2-dicapryroyl-3-eicosapentaenoyl-*sn*-glycerol.

Figure 4 shows TAG regioisomers and enantiomers identified by chiral LC from *K. polysporus*. The number of TAGs identified by chiral LC was higher than NARP-LC, which is opposite to what has so far been claimed [24]. We assume that it is caused by the column that we used, which provided better separation. The relative abundance of TAG enantiomers and regioisomers in yeast TAGs is listed in Table 2. The retention times in the TICs and the identities of the $[M+H-RCOO]^+$ fragments used for NLS were used for qualitative identification of the TAG regioisomers and enantiomers. If the enantiomers were not separated, as in the case of saturated TAGs, i.e. PPS and SPP, these molecular species were not quantified because they were also not separated by chiral LC and they give identical mass spectra, see above. In strains producing palmitoleic acid in amounts >25% of total FAs, this acid or an unsaturated FA is bound in *sn*-1. In strains containing from 10 to 25% palmitoleic acid in total FAs, this acid is bound in *sn*-3, saturated FA being bound in *sn*-1.

4 Concluding remarks

The results of NARP-LC and chiral LC analyses indicated that the major difference between the TAGs lies in the length and unsaturation of fatty acyls, and in abundances of regioisomers and enantiomers. The TAG with the highest abundance was triolein (OOO), which was found in all strains in relative amounts higher than 5%, closely followed by *sn*-OPoO (also identified in all strains). Three TAGs (PoPoPo, *sn*-PoOPo, and *sn*-OOPo) had at least one potentially nutritionally important palmitoleic acid in the molecule; this is illustrated also by Table 1 presenting the content of FA. The strain *K. polysporus* producing the highest amounts of palmitoleic acid

produces more than two-thirds of TAGs containing palmitoleic acid. NLS provided a platform for analyzing individual lipid molecular species directly from appropriate TAGs.

Both LC methods, i.e. NARP-LC and chiral LC, could serve for the separation of TAGs from different sources. It is thus possible to identify nearly 50% of all theoretically possible TAGs (i.e. 94 identified out of the 216 theoretically possible). For comparison, Francois et al. [45] identified 324 TAGs in menhaden fish oil. As shown also by the study described in [24] (which identified 19 or 26 TAGs out of the 64 theoretically possible ones), chiral LC makes it possible to increase significantly the number of identified TAGs including regioisomers and enantiomers, and thereby aids in elucidating the biosynthetic and metabolic processes in living cells.

Monounsaturated TAGs are valuable precursors of lipid-based products important for nutrition, cosmetics, or biofuels. TAG-component monoenoic FAs with chain lengths up to C_{19}, especially palmitoleic acid, are known to impart excellent oxidative stability and cold-flow properties to fuels [46]. Many of the yeast strains under study have TAG profiles containing significant amounts of palmitoleic acid and could serve as its source. Our APCI-MS method is well suited for the direct determination of TAG profiles and for comparing the production of lipids after a genetic manipulation or a change in yeast cultivation conditions.

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